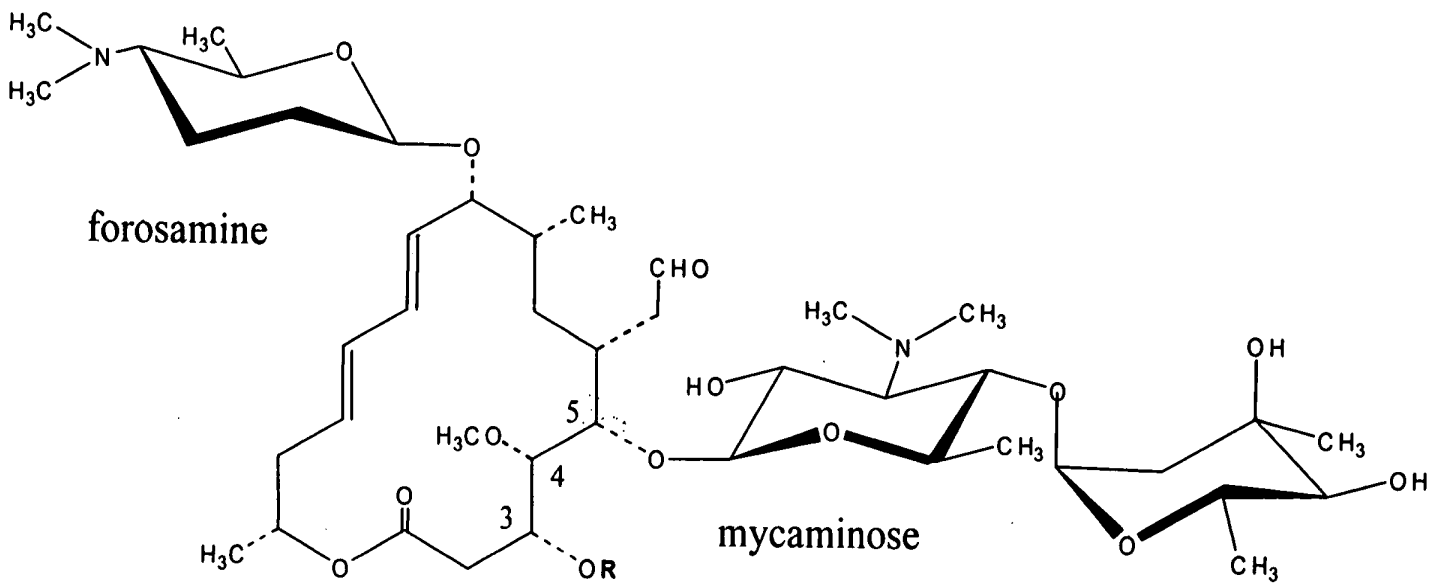
OIPE
SEP 23 2004
PATENT & TRADEMARK OFFICE



R=H spiramycin I

R= $COCH_3$ spiramycin II

R= $COCH_2CH_3$ spiramycin III

FIG. 1

Insert pOS49.1
Insert pSPM5
Insert pSPM7
Chromosome
ATCC23877
10 kb

FIG. 2

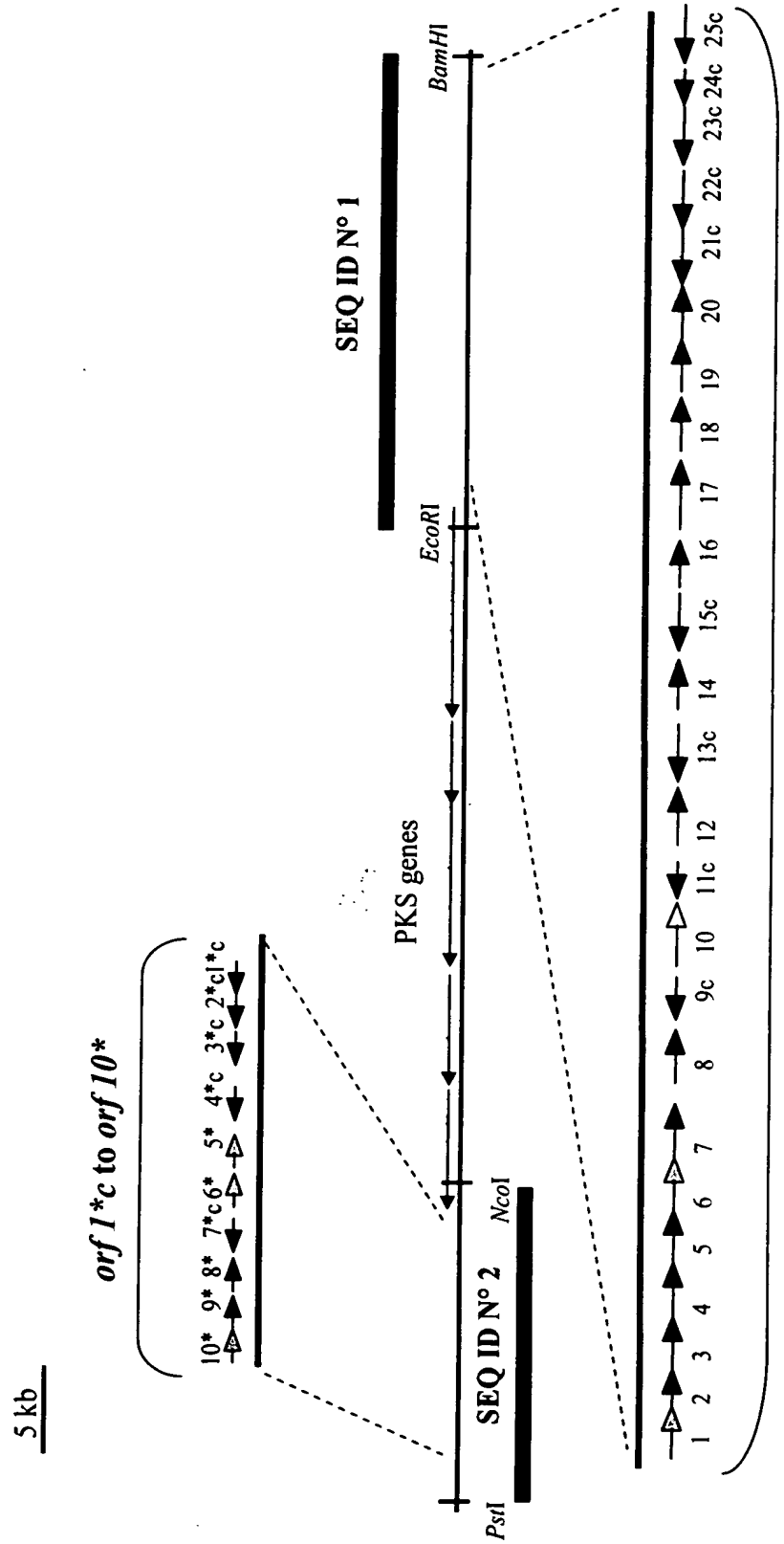

5 kb
orf 1*c to orf 10*
10* 9* 8* 7*c 6* 5* 4*c 3*c 2*c 1*c
SEQ ID N° 1
SEQ ID N° 2
PKS genes
PstI
NcoI
EcoRI
BamHI
1 2 3 4 5 6 7 8 9c 10 11c 12 13c 14 15c 16 17 18 19 20 21c 22c 23c 24c 25c
orf 1 to orf 25c

FIG. 3

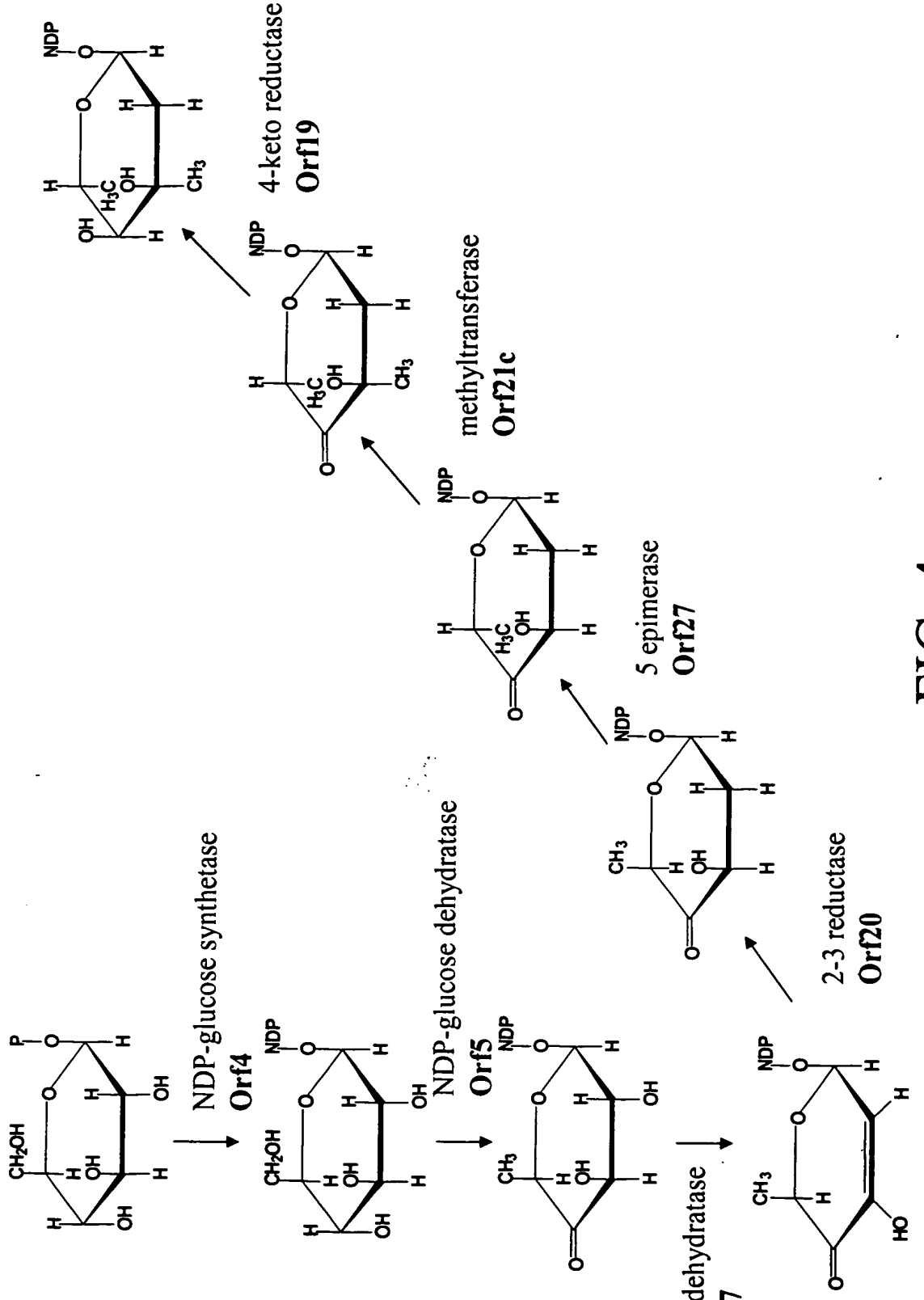

NDP-glucose synthetase
Orf4
NDP-glucose dehydratase
Orf5
2-3 dehydratase
Orf7
2-3 reductase
Orf20
5 epimerase
Orf27
methyltransferase
Orf21c
4-keto reductase
Orf19

FIG. 4

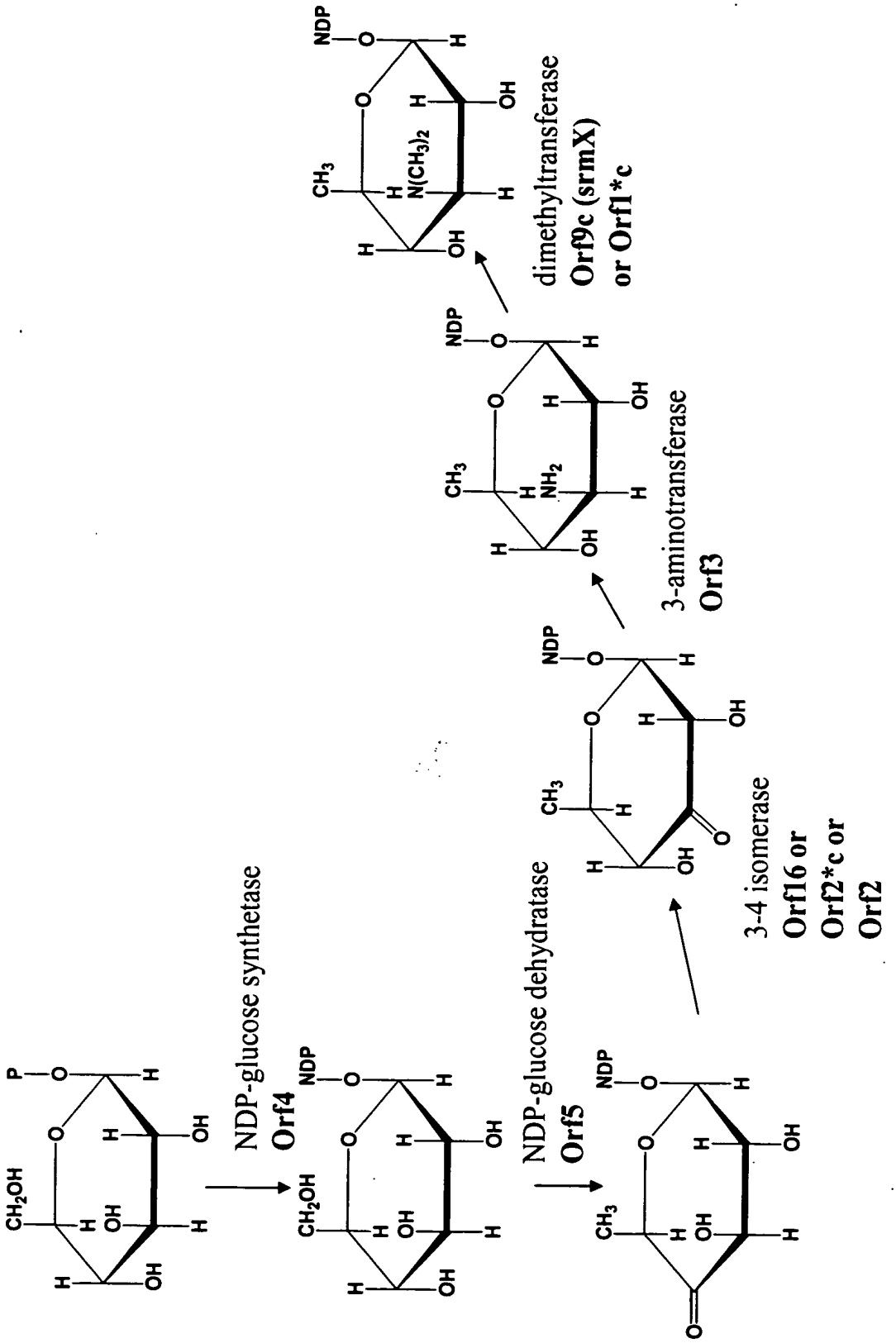

NDP-glucose synthetase
Orf4
NDP-glucose dehydratase
Orf5
3-4 isomerase
Orf16 or
Orf2*c or
Orf2
3-aminotransferase
Orf3
dimethyltransferase
Orf9c (srmX)
or Orf1*c

FIG. 5

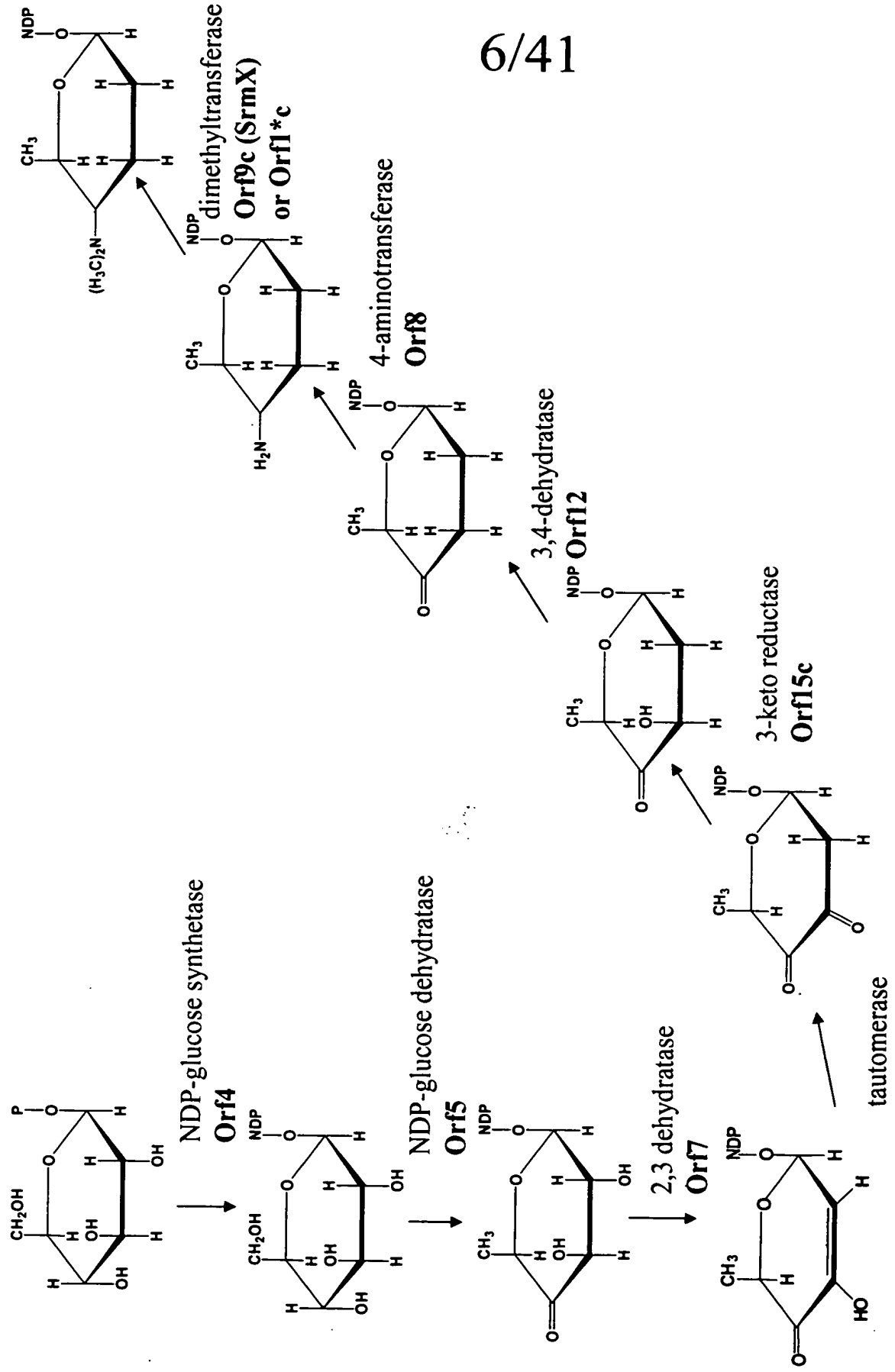

NDP-glucose synthetase
Orf4
NDP-glucose dehydratase
Orf5
2,3 dehydratase
Orf7
tautomerase
Orf2*c
or Orf16
3-keto reductase
Orf15c
3,4-dehydratase
Orf12
4-aminotransferase
Orf8
dimethyltransferase
Orf9c (SrmX)
or Orf1*c

FIG. 6

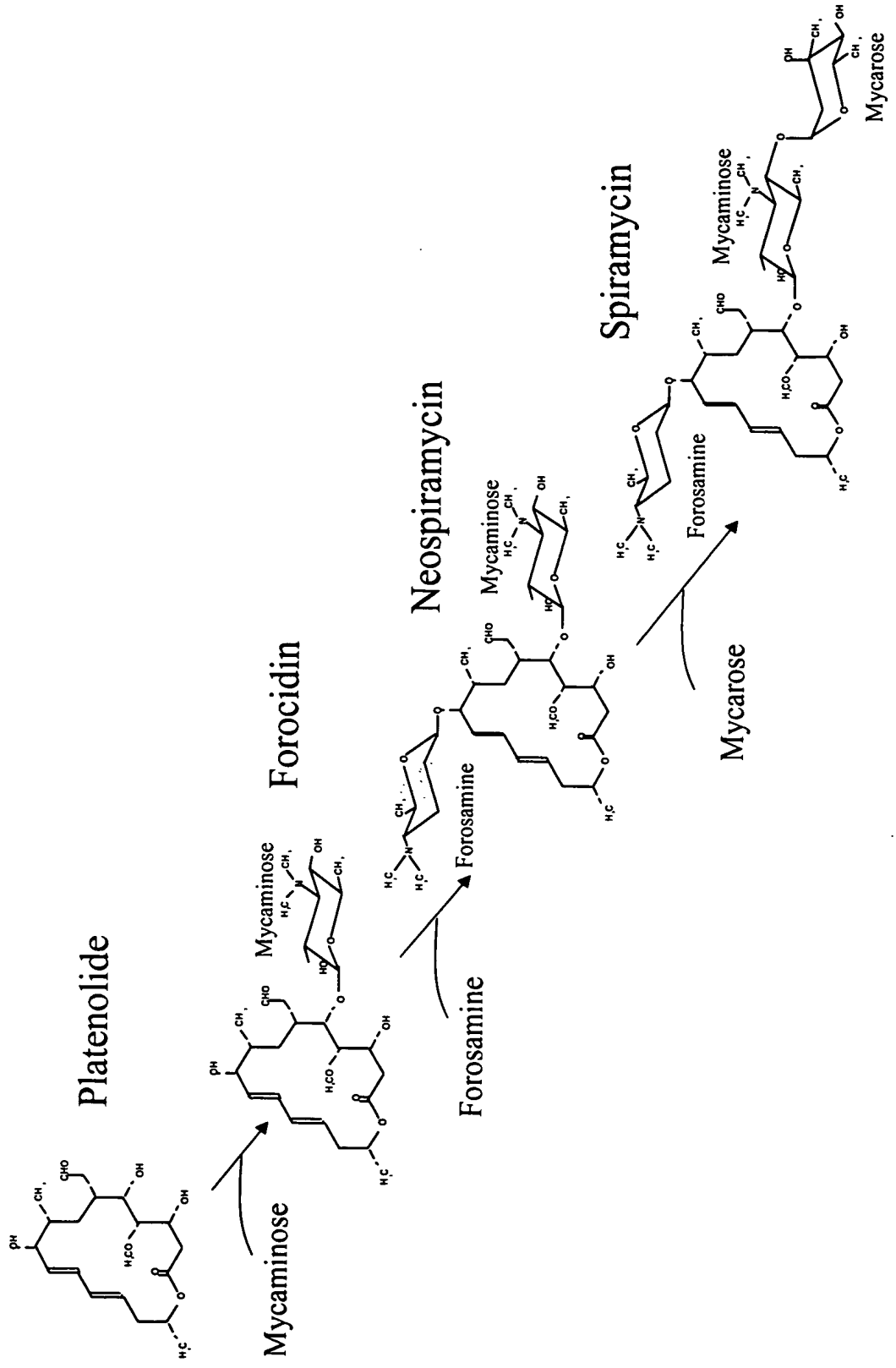

Platenolide
Mycaminose
Forocidin
Mycaminose
Forosamine
Neospiramycin
Mycaminose
Forosamine
Mycarose
Spiramycin
Mycaminose
Mycarose

FIG. 7

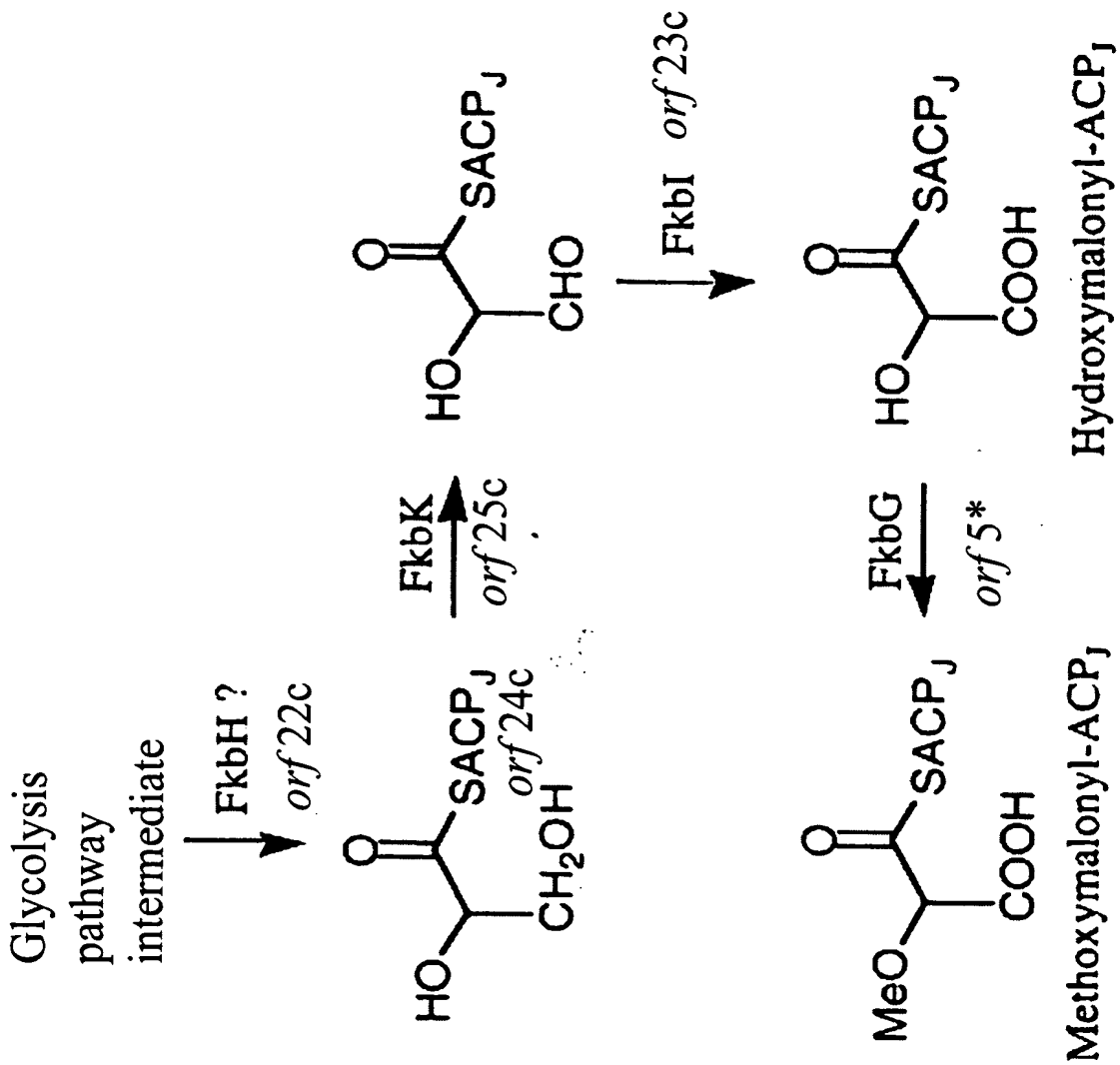

Glycolysis pathway intermediate
FkbH ?
orf 22c
SACP$_J$
CH$_2$OH
HO
orf 24c
FkbK
orf 25c
SACP$_J$
CHO
HO
FkbI orf 23c
SACP$_J$
COOH
HO
Hydroxymalonyl-ACP$_J$
FkbG
orf 5*
SACP$_J$
COOH
MeO
Methoxymalonyl-ACP$_J$

FIG. 8

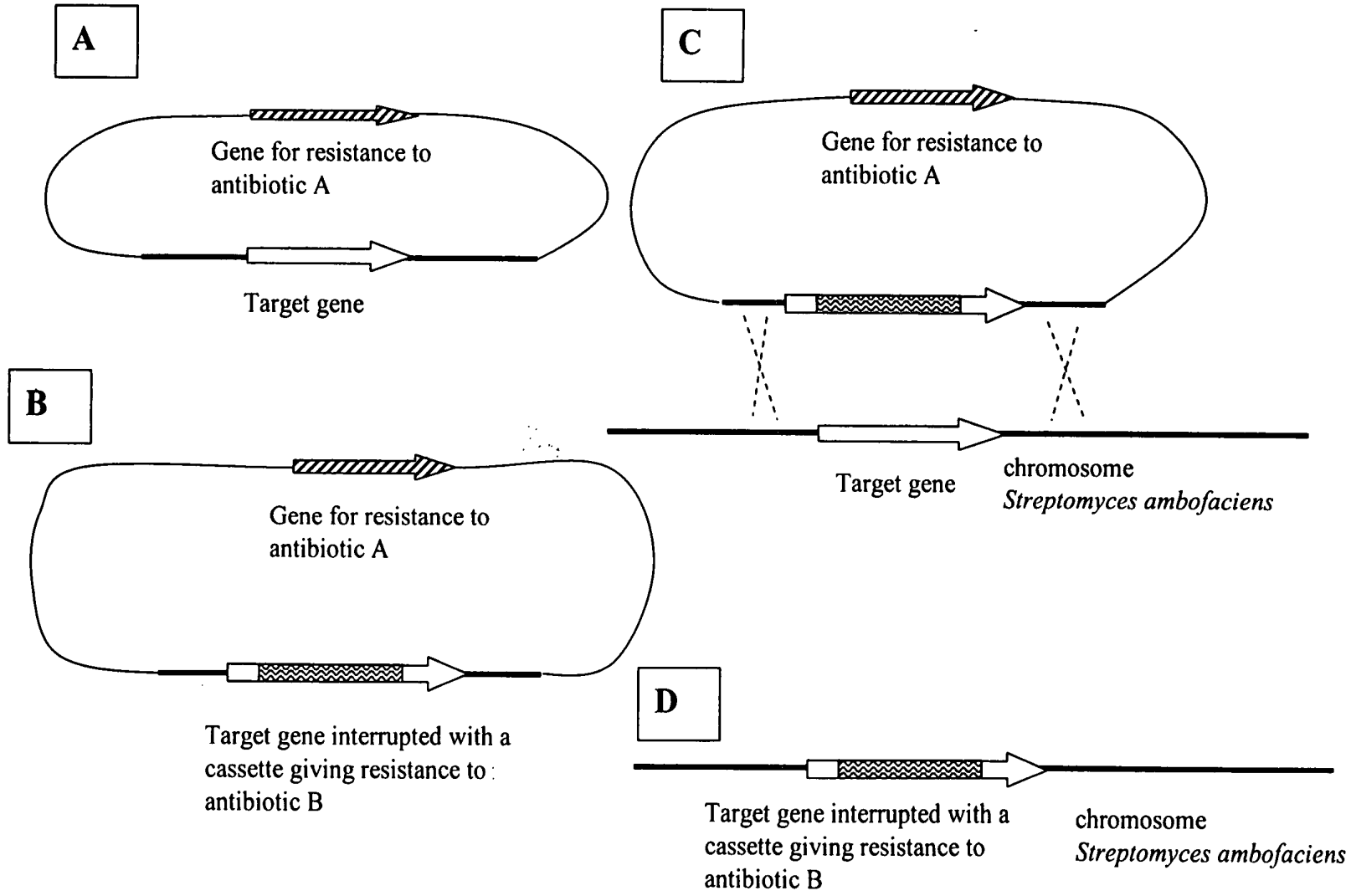

A
Gene for resistance to antibiotic A
Target gene
B
Gene for resistance to antibiotic A
Target gene interrupted with a cassette giving resistance to antibiotic B
C
Gene for resistance to antibiotic A
Target gene
chromosome
Streptomyces ambofaciens
D
Target gene interrupted with a cassette giving resistance to antibiotic B
chromosome
Streptomyces ambofaciens

FIG. 9

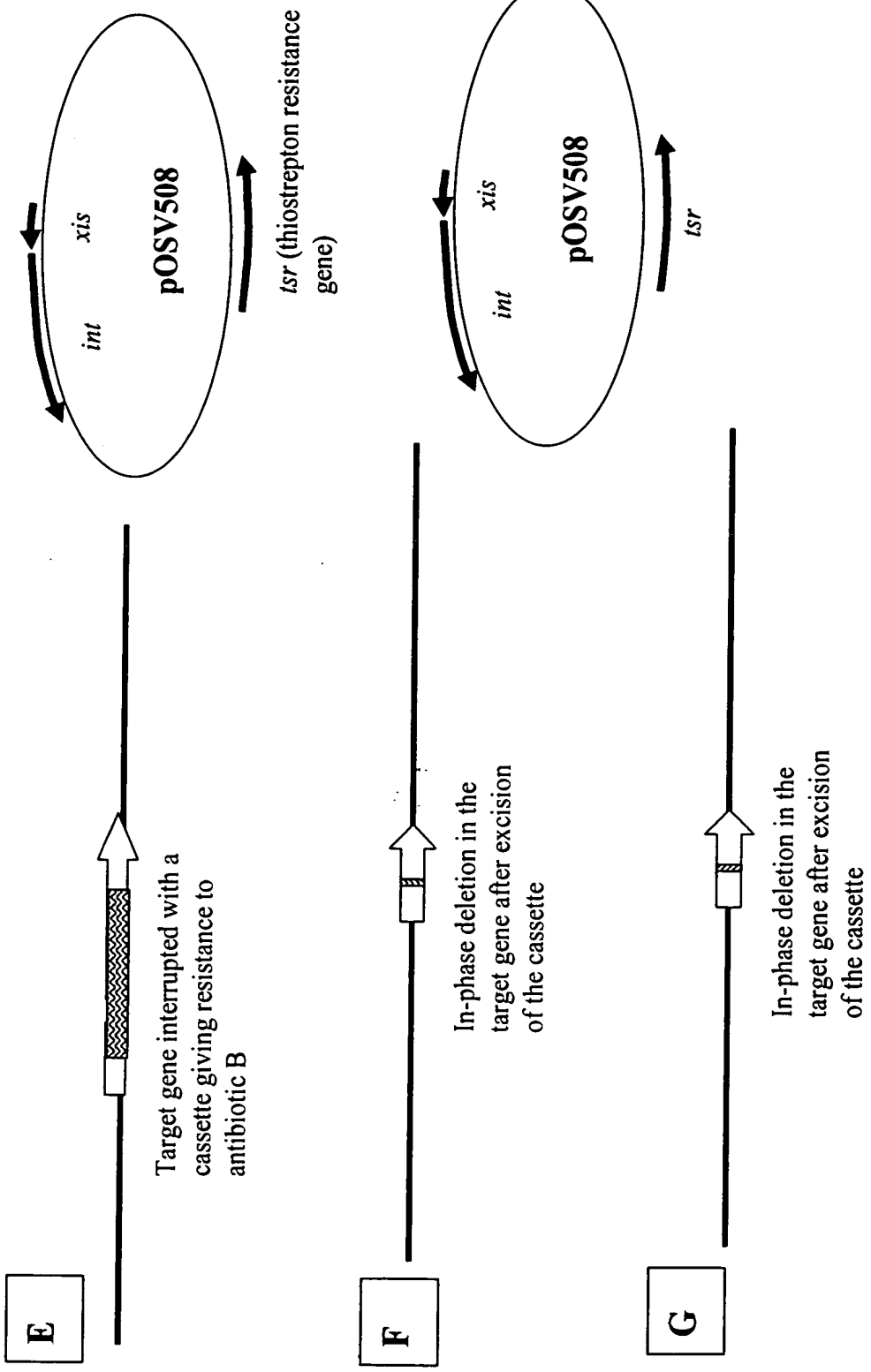

E
Target gene interrupted with a cassette giving resistance to antibiotic B
int
xis
pOSV508
tsr (thiostrepton resistance gene)
F
In-phase deletion in the target gene after excision of the cassette
int
xis
pOSV508
tsr
G
In-phase deletion in the target gene after excision of the cassette

FIG. 10

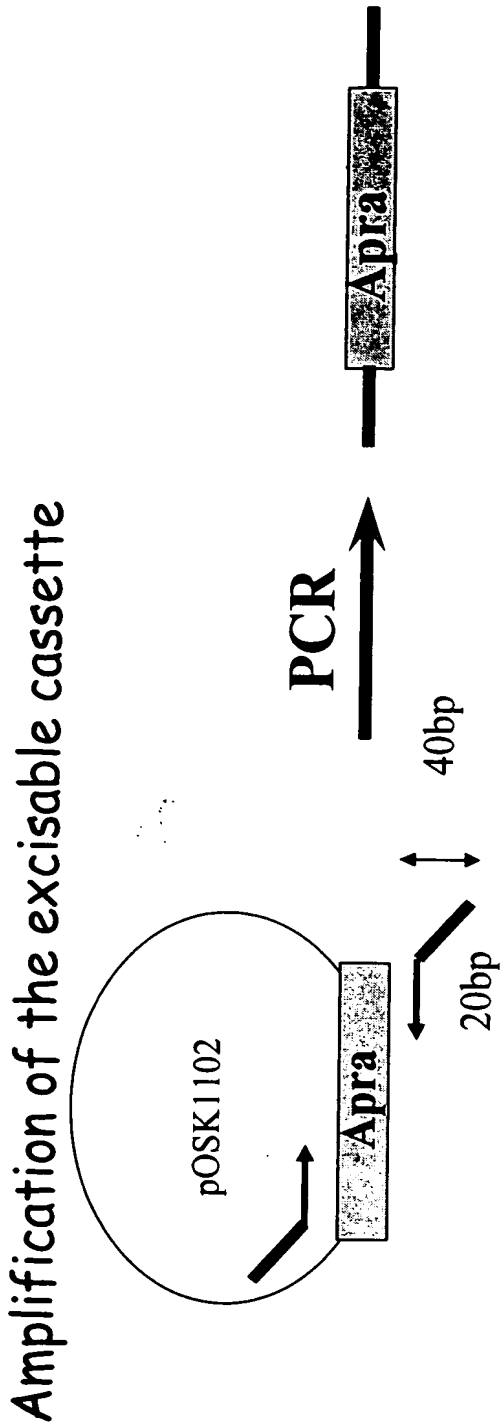

Amplification of the excisable cassette
pOSK1102
Apra
20bp
40bp
PCR
Apra

FIG. 11

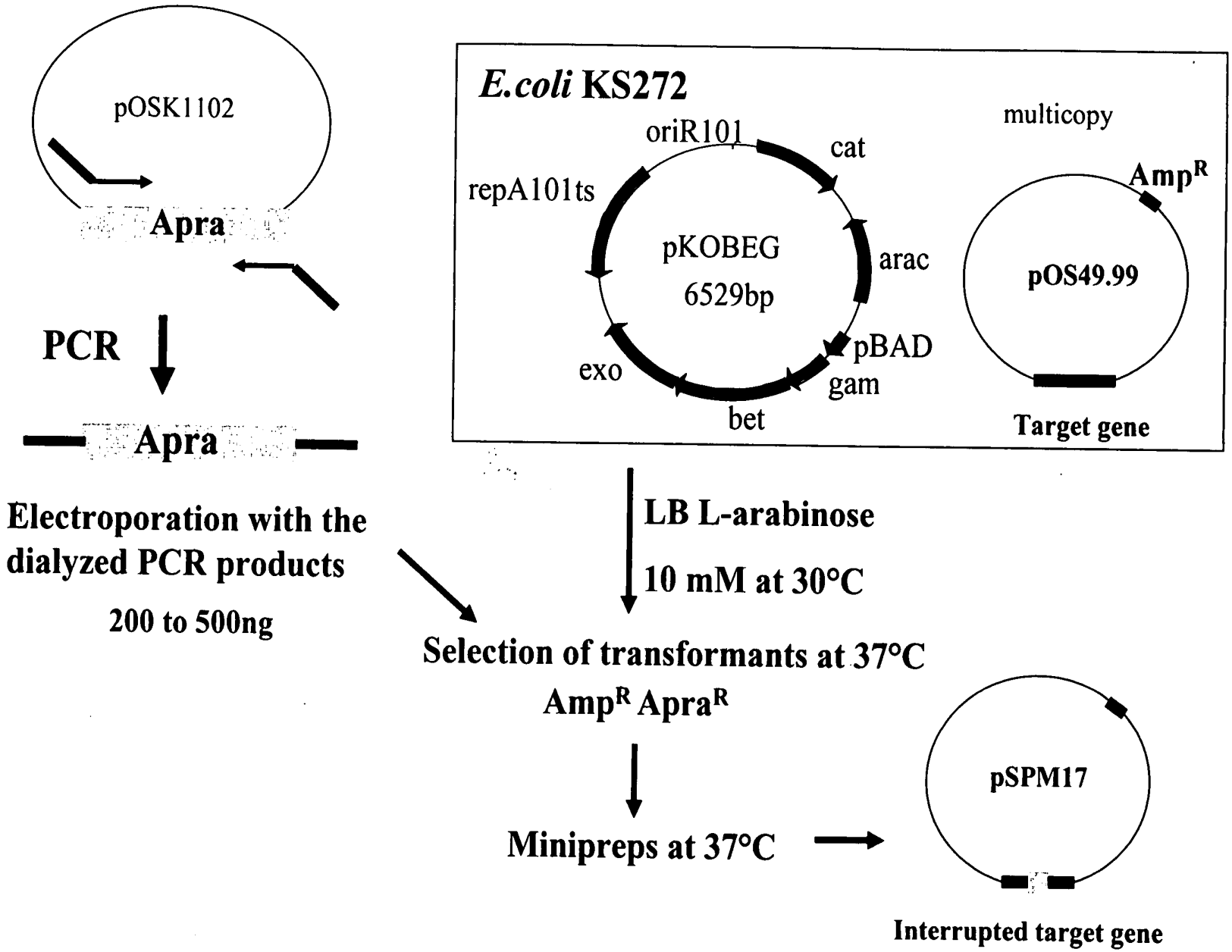

pOSK1102
Apra
PCR
Apra
Electroporation with the dialyzed PCR products
200 to 500ng
E.coli KS272
oriR101
cat
repA101ts
pKOBEG
6529bp
arac
pBAD
exo
gam
bet
multicopy
Amp^R
pOS49.99
Target gene
LB L-arabinose
10 mM at 30°C
Selection of transformants at 37°C
Amp^R Apra^R
Minipreps at 37°C
pSPM17
Interrupted target gene

FIG. 12

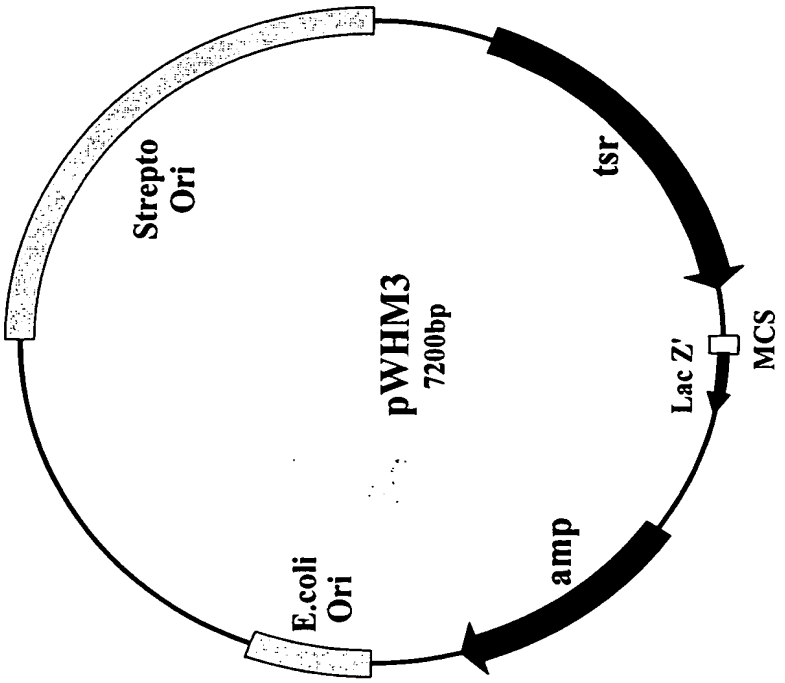


FIG. 13

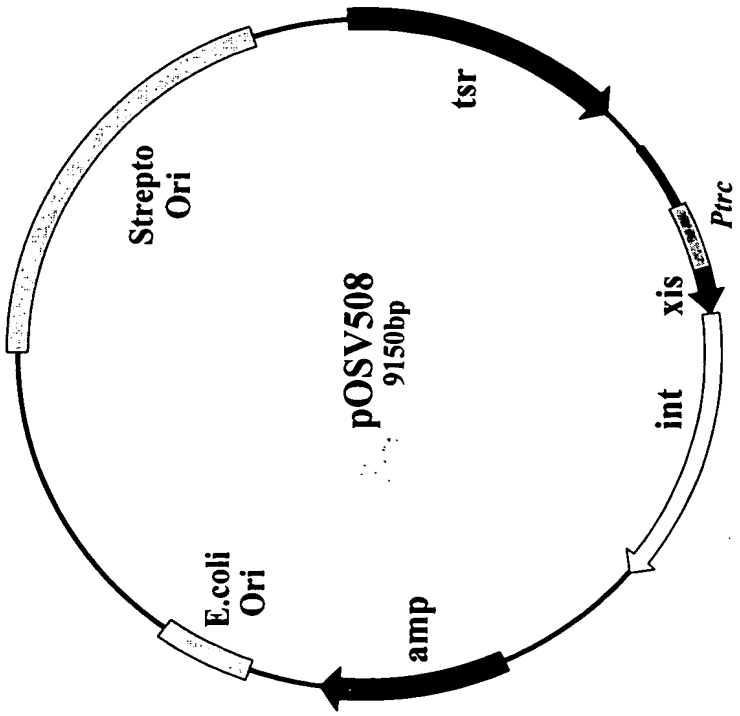


FIG. 14

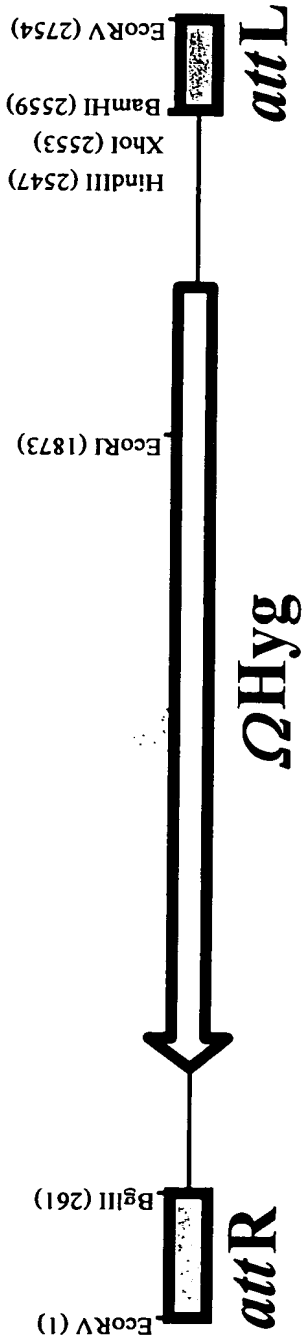

EcoRV (1)
attR
BglII (261)
ΩHyg
EcoRI (1873)
HindIII (2547)
XhoI (2553)
BamHI (2559)
attL
EcoRV (2754)

FIG. 15

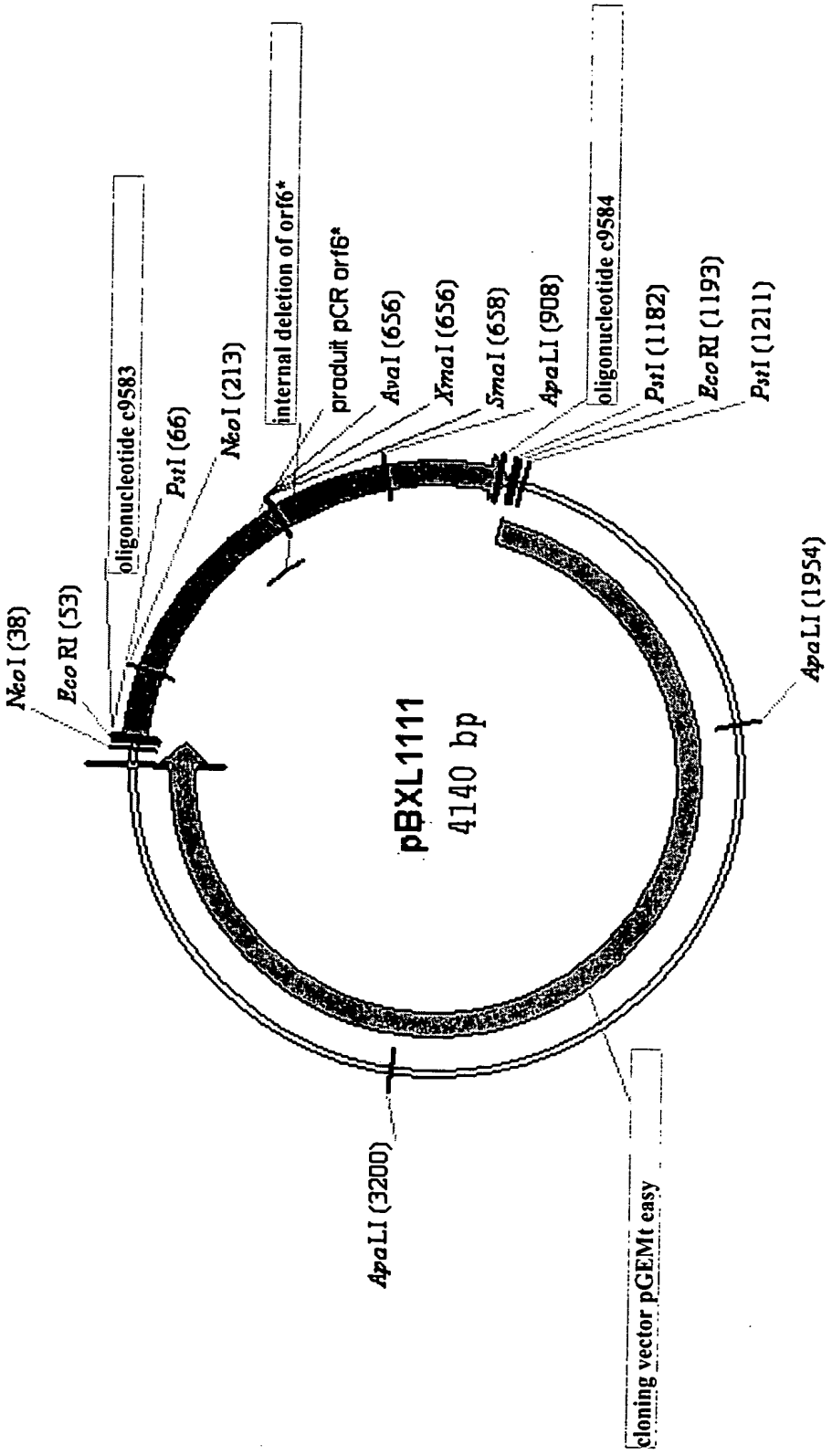

pBXL1111
4140 bp
NcoI (38)
EcoRI (53)
oligonucleotide c9583
PstI (66)
NcoI (213)
internal deletion of orf6*
produit pCR orf6*
AvaI (656)
XmaI (656)
SmaI (658)
ApaLI (908)
oligonucleotide c9584
PstI (1182)
EcoRI (1193)
PstI (1211)
ApaLI (1954)
ApaLI (3200)
cloning vector pGEMt easy

FIG. 16

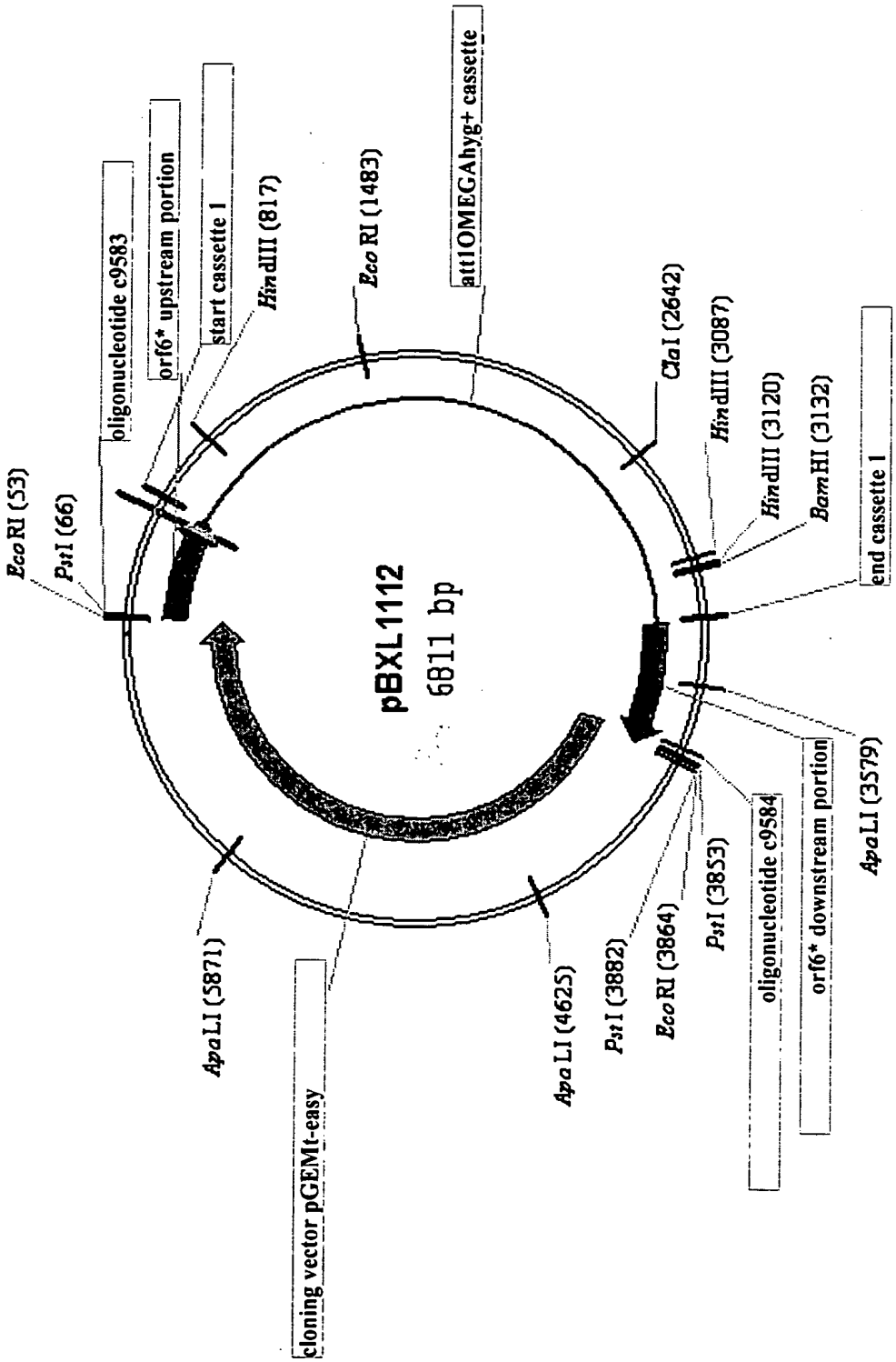

pBXL1112
6811 bp
EcoRI (53)
PstI (66)
oligonucleotide c9583
orf6* upstream portion
start cassette 1
HindIII (817)
EcoRI (1483)
attIOMEGAhyg+ cassette
ClaI (2642)
HindIII (3087)
HindIII (3120)
BamHI (3132)
end cassette 1
ApaLI (3579)
orf6* downstream portion
oligonucleotide c9584
PstI (3853)
EcoRI (3864)
PstI (3882)
ApaLI (4625)
cloning vector pGEMt-easy
ApaLI (5871)

FIG. 17

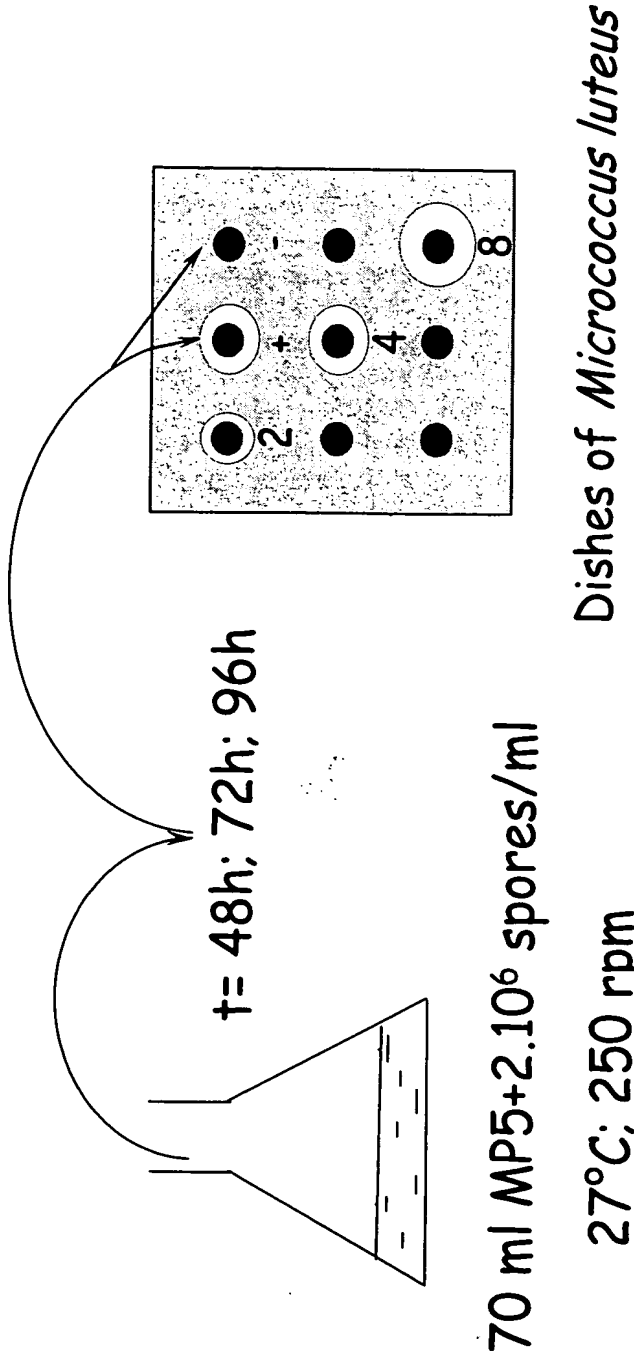

70 ml MP5+2.10⁶ spores/ml
27°C; 250 rpm
t= 48h; 72h; 96h
Dishes of Micrococcus luteus
37°C, 24-48h
-
+
2
4
8

FIG. 18

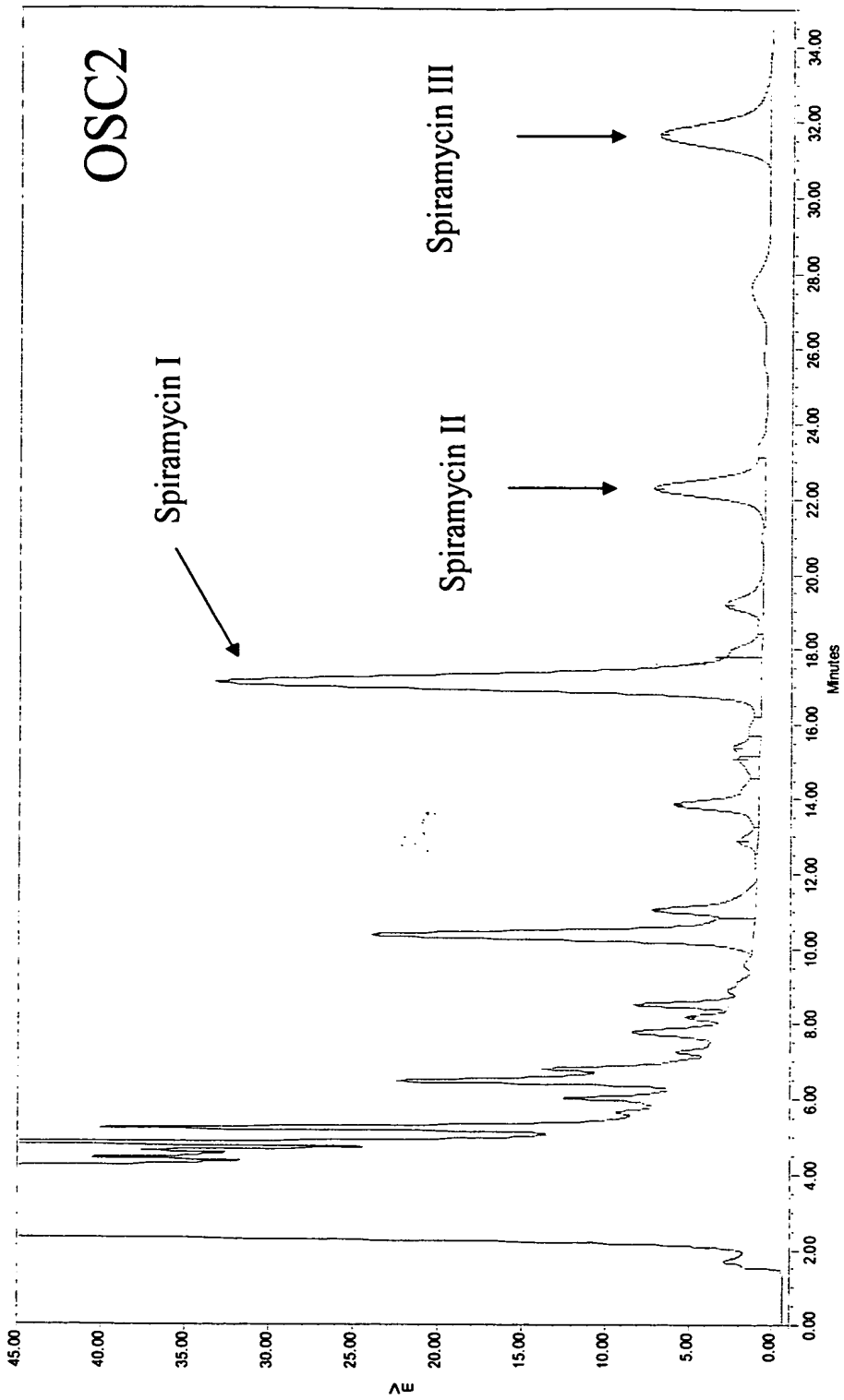

OSC2
Spiramycin I
Spiramycin II
Spiramycin III
mV
Minutes
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00

FIG. 19

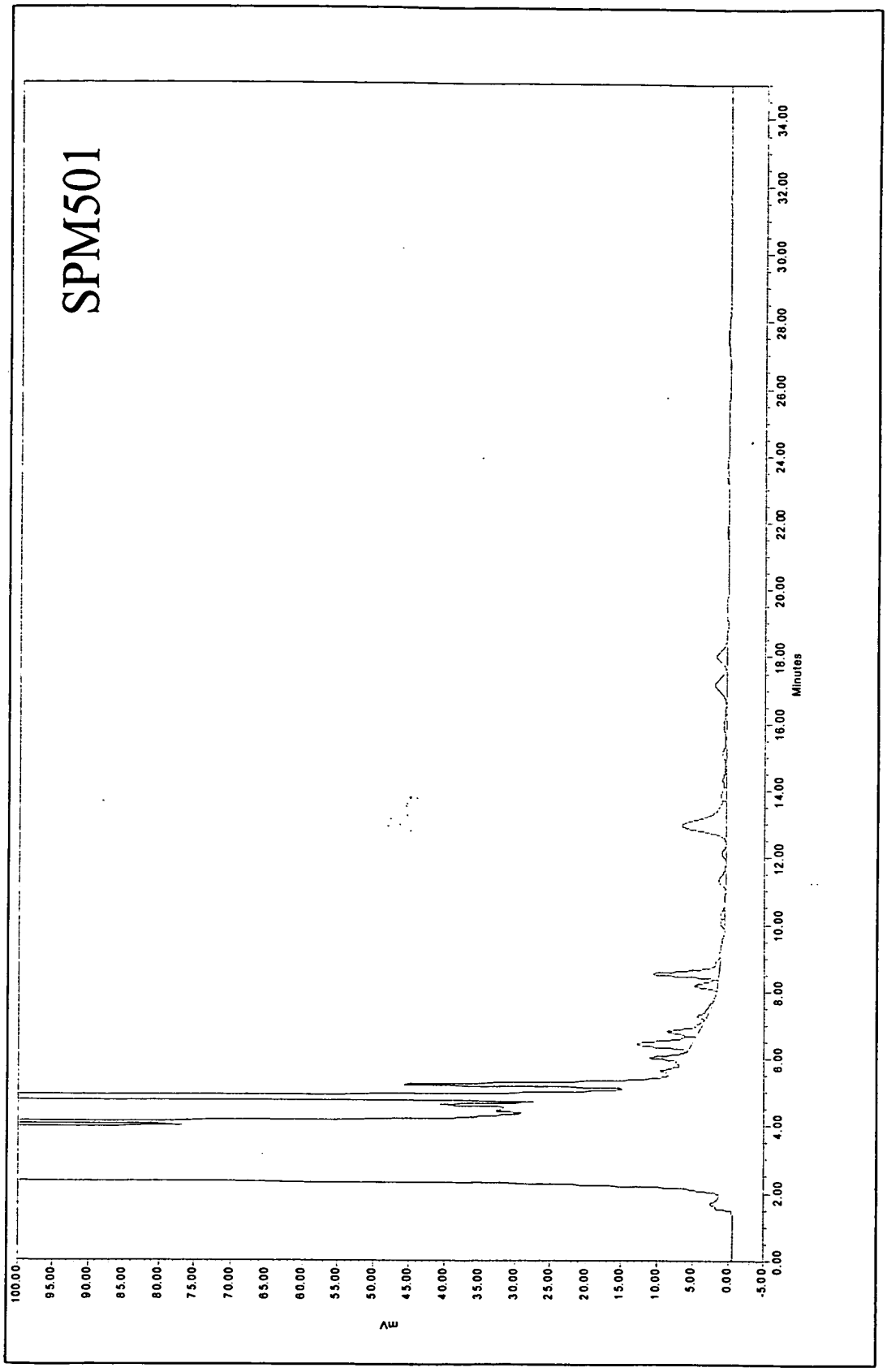

SPM501
mV
100.00
95.00
90.00
85.00
80.00
75.00
70.00
65.00
60.00
55.00
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
Minutes

FIG. 20

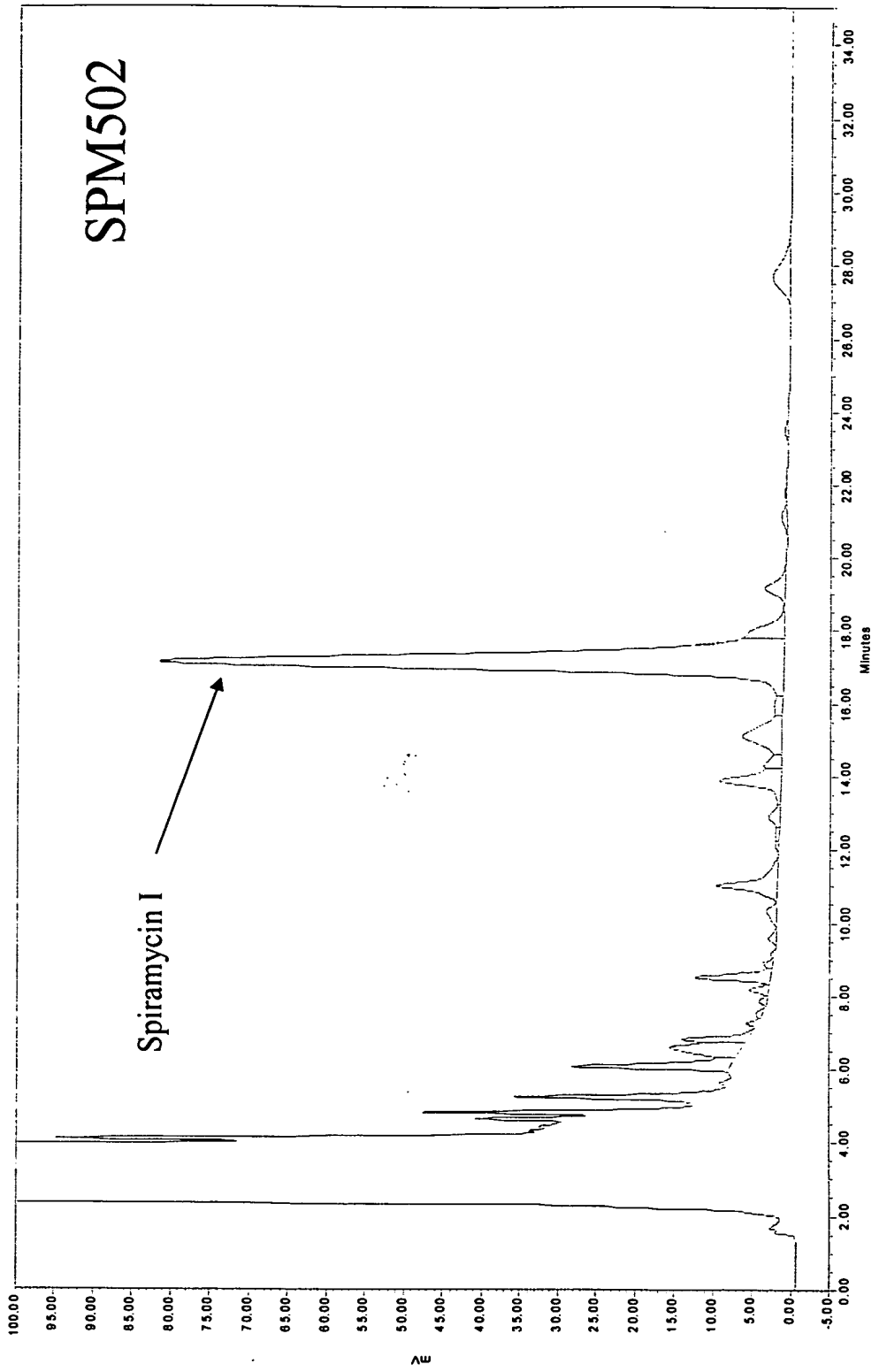

SPM502
Spiramycin I
mV
Minutes
100.00
95.00
90.00
85.00
80.00
75.00
70.00
65.00
60.00
55.00
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00

FIG. 21

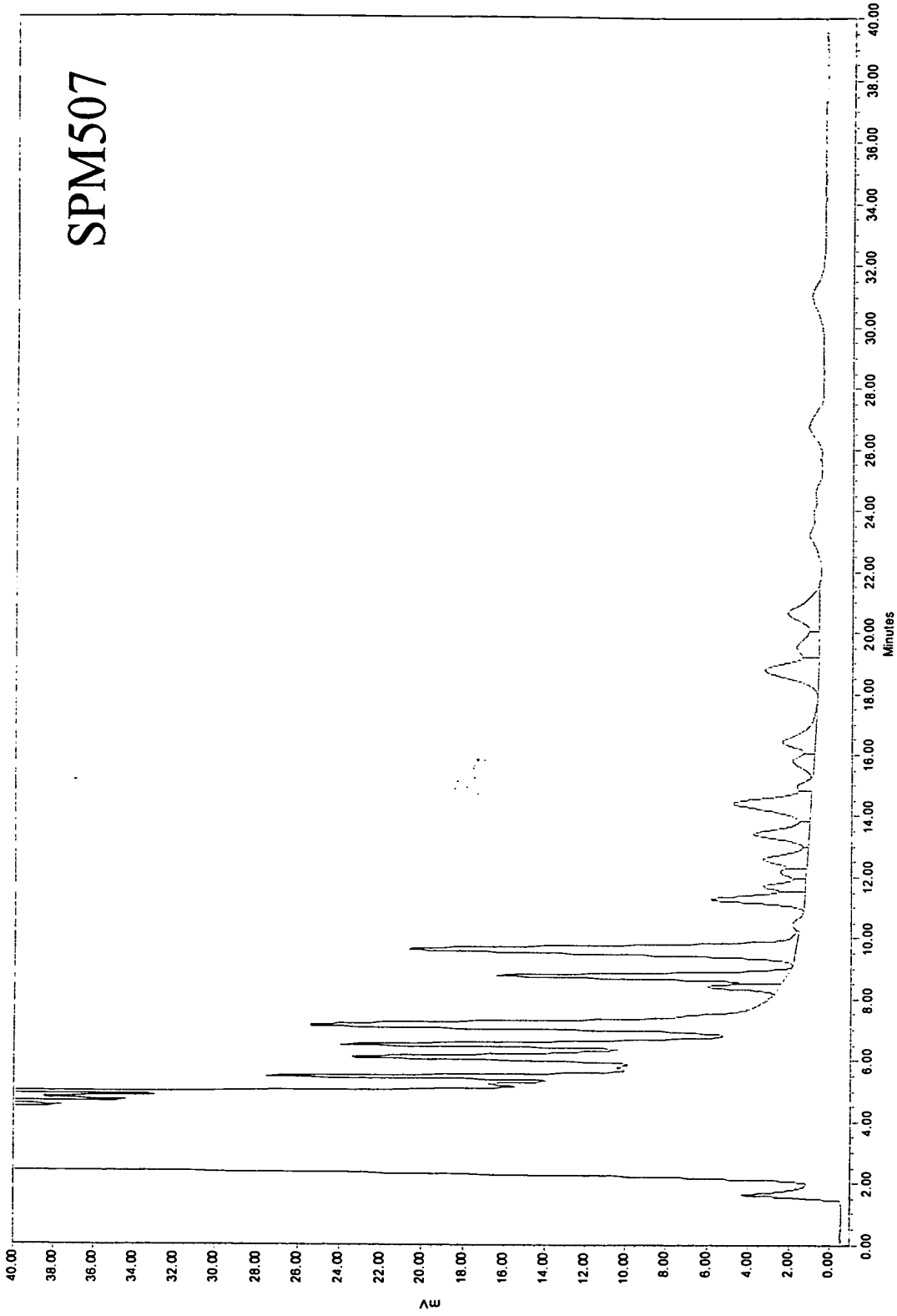

SPM507
mV
Minutes
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
36.00
38.00
40.00

FIG. 22

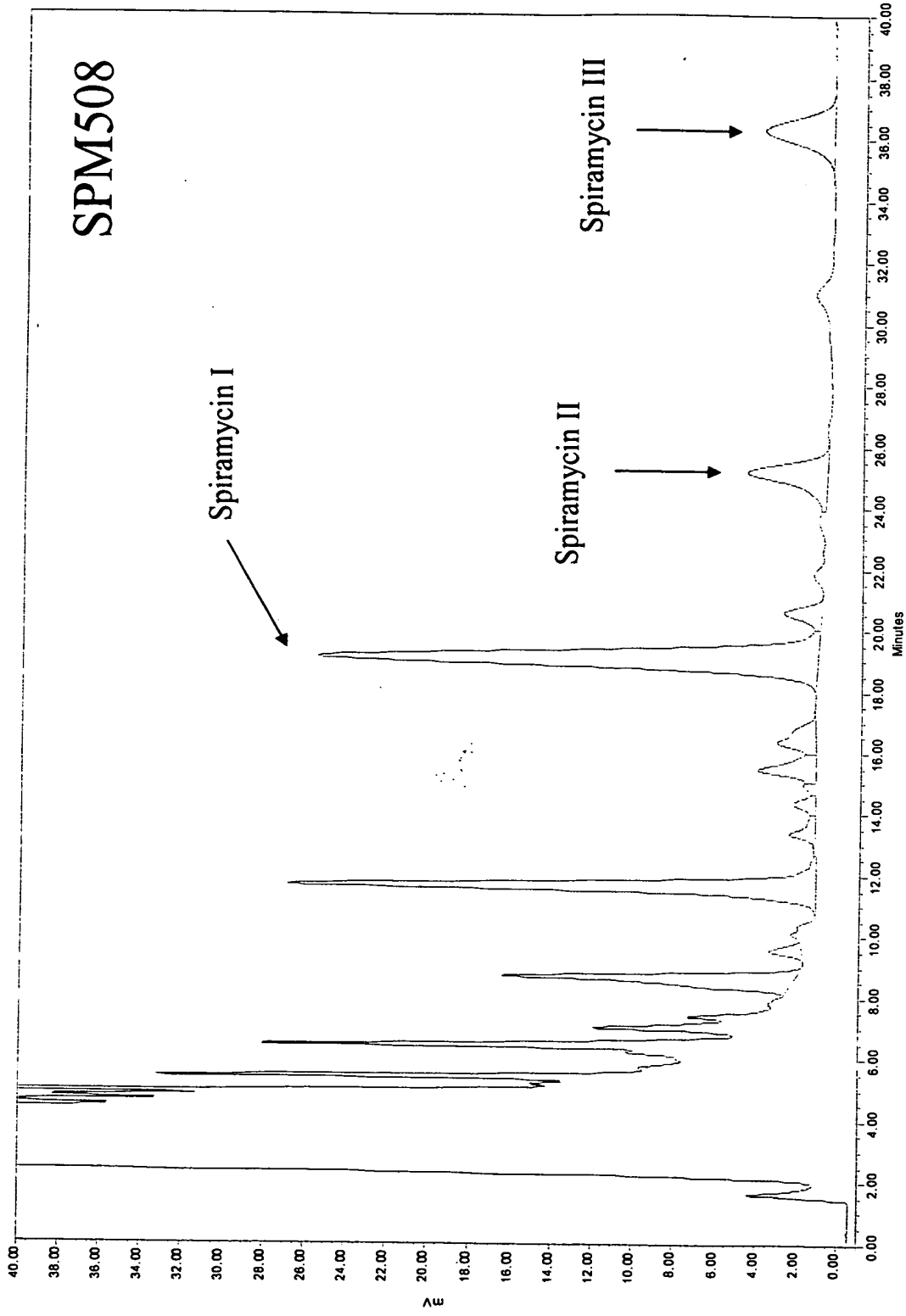

SPM508
Spiramycin I
Spiramycin II
Spiramycin III
mV
Minutes
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
36.00
38.00
40.00

FIG. 23

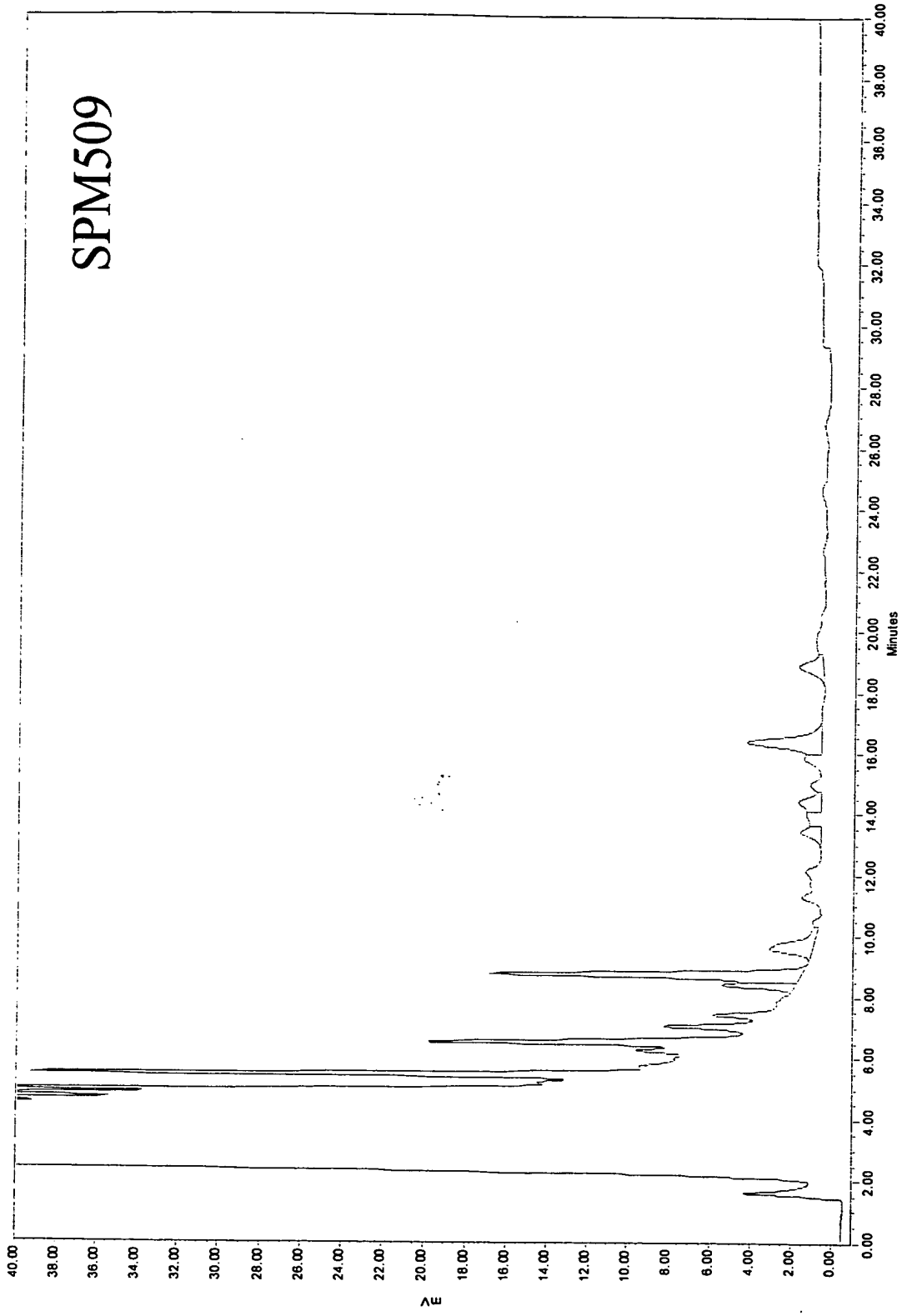

SPM509
mV
Minutes
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
36.00
38.00
40.00

FIG. 24

```
FASTA (3.42 Sept 2001) function [optimized, BL50 matrix (15:-5)]
ktup: 2 join: 37, opt: 25, open/ext: -10/-2, width:  16

Smith-Waterman score: 1467;  63.130% identity (66.480% ungapped) in
377 aa overlap (3-367:11-380)

                          10        20        30        40
orf3           MTIPFLD-----AGAGYRELRAEIDAALQRVSASGRYLLDAELAAFEEEFAA
                 .:: :     :..:   ...:: .:: ::.: ::::: :::::::::.::
tylB    MTGLPRPAVRVPFHDLRDVHAATG---VESEIGGALLRVAARGRYLLGAELAAFEERFAE
               10        20            30        40        50

         50        60        70        80        90        100
orf3    YCDNDHCVAVGSGCDALELSLRALDIGPGDEVVVPAHTFIGTWLAVSATGARPVAV----
        :: : ::::::::: :  .:.: :: .: ::::.::.::::..:::::::::: :: :
tylB    YCGNAHCVAVGSGLDDARLALWALGVGEGDEVIVPSHTFIASWLAVSATGATPVPVEPGD
         60        70        80        90        100       110


              110       120       130       140       150       160
orf3    --DPTPDGLSLDPALVEAALTPRTRALMPVHLHGHPADLDPLLAIAGRHGLAVVEDAAQA
          .: : .. :::  .:::::::::::.::::.:::.::::. :.:  ::::::::::::
tylB    PGEPGPGAFLLDPDRLEAALTPRTRAVMPVHLYGHPVDLDPVGAFAEPHGLAVVEDAAQA
        120       130       140       150       160       170

              170       180       190       200       210       220
orf3    HGARYRGRRIGSGHVVAFSFYPGKNLGAMGDGGAVVTGDSGVAERIRLLRNCGSREKYRH
          :::::::::::: .:::::::::::::.:::::::::.:  .:.:.:::::: :.:::::::
tylB    T-ARYRGRRIGSGHRTAFSFYPGKNLGALGDGGAVVTSDPELADRLRLLRNYGAREKYRH
         180       190       200       210       220       230

              230       240       250       260       270       280
orf3    EVRSTHSRLDEFQAAVLRAKLPRLDAWNARRAGTAERYGRALGPVPQIAVPVTAPWADPV
        : :.:.:::::.::::: .::: ::::::.::   : :::.::. .: ..::  .  :.::
tylB    EERGTNSRLDELQAAVLSVKLPYLDAWNTRRREIAARYGEALAGLPGVTVP-EGRVAEPV
         240       250       260       270       280       290

              290       300       310       320       330       340
orf3    WHLYVIRCAERDELRRRLERAGVQTLIHYPVPPHRSPAYADDPAGA-PAGTHPLSERLAA
        :: ::.:   ::.::::: .::::.::.::::  : : :::   ::  :::  : .:::::.
tylB    WHQYVLRSPYRDRLRRRLAEAGVETLVHYPVAVHASGAYAG--AGPCPAGGLPRAERLAG
          300       310       320       330       340       350

               350       360       370       380
orf3    QSLSLPLGPHLGEDEARAVVAAVRAASAGLAAYPTPDGQRFPLVTEKR
        . ::::.::::: .. ...:.::::..:.
tylB    EVLSLPIGPHLPDEAVEVVIAAVQSAALDSWEEGP
            360       370       380
```

FIG. 25

Comparison of:
(A) SRMD=Query

(B) MDMA=Sbjct

>gi|320536|pir||A60725 midecamycin-resistance protein mdmA - Streptomyces mycarofaciens
Length = 271

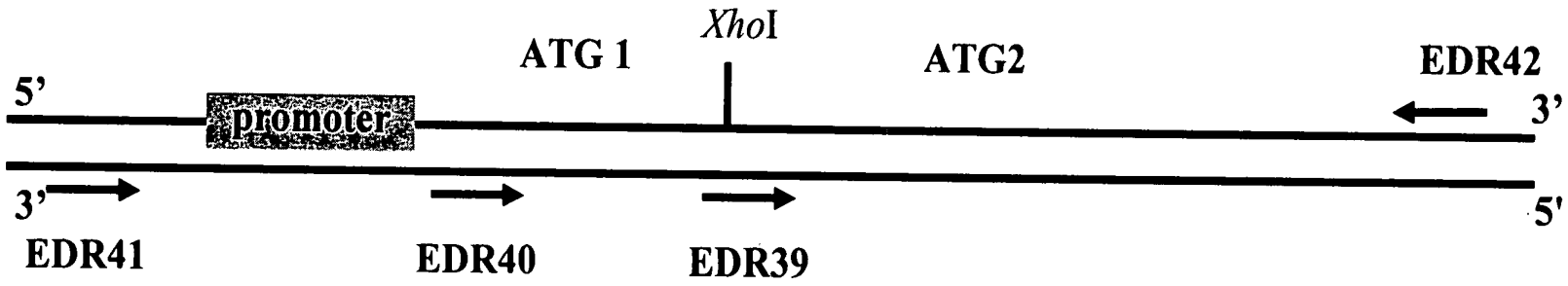
Score = 380 bits (975), Expect = e-104
Identities = 211/269 (78%), Positives = 232/269 (85%)

```
(A) Query: 7   LPPQSVSAPADSRSTARREWGQNFFRTAAAACRFSAQLDGSDTIPPDSPNDLMTVEIGAG 66
               + P S SAPA SRSTARRE GQNFFR+AAAACRFS QLD      P S  D++TVEIGAG
(B) Sbjct: 1   MSPISASAPAASRSTARRELGQNFFRSAAAACRFSDQLDAFCADLPGSLADVLTVEIGAG 60

(A) Query: 67  SGRVTKVLASPGTPLLAVEIDPRWARRLAAESLPDVTVVNEDFLTLQLPGQPVRLIGNLP 126
               SGRVTK LAS G  LLAVEID  WARRL AESLPDVTVVNEDFL LQLP QP+RLIGNLP
(B) Sbjct: 61  SGRVTKALASAGRSLLAVEIDAYWARRLTAESLPDVTVVNEDFLNLQLPRQPIRLIGNLP 120

(A) Query: 127 FVTGTRMLRRCLDMGPARMRQGVFLLQREYVGKRTGAWGGNLFNAQWEPWYSFDRGLAFS 186
               FV+GT++LRRCL++GP RM Q VFLLQREYVGKRTGAWGGNLFNAQWEPWY+F+ GLAFS
(B) Sbjct: 121 FVSGTKILRRCLELGPNRMCQAVFLLQREYVGKRTGAWGGNLFNAQWEPWYTFEGGLAFS 180

(A) Query: 187 RQDFTPVPRADTQTLMVAPHRRPSVPWREKAAYQRFVQRVFDTGQMTVGDAARKVLRRGH 246
               R +F+PVPRADTQTL+V P RRPSVPWRE+  YQRF Q++FDTGQMT+G+AARKVLRRGH
(B) Sbjct: 181 RNEFSPVPRADTQTLVVMPRRRPSVPWRERTDYQRFTQQIFDTGQMTIGEAARKVLRRGH 240

(A) Query: 247 AQFVRGAGVRPADRVKDLTVPEWTALFRA 275
               AQFVR AGVRPADRVKDLTV +W ALFRA
(B) Sbjct: 241 AQFVRSAGVRPADRVKDLTVRDWAALFRA 269
```


FIG. 26

phase 1 (att1): atc**GCGCGCTTCGTTCGGGACGAAGAGGT**agat
phase 2 (att2): atcg**GCGCGCTTCGTTCGGGACGAAGAGGT**agat
phase 3 (att3): atcgc**GCGCGCTTCGTTCGGGACGAAGAGGT**agat

FIG. 27



ATG 1: ATG located in the most 5' position
ATG 2: ATG in the most 3' position

EDR39-EDR42 → " short *orf10* " pSPM523

EDR40-EDR42 → " long *orf10* " pSPM524

EDR41-EDR42 → " pro *orf10* " pSPM525

FIG. 28

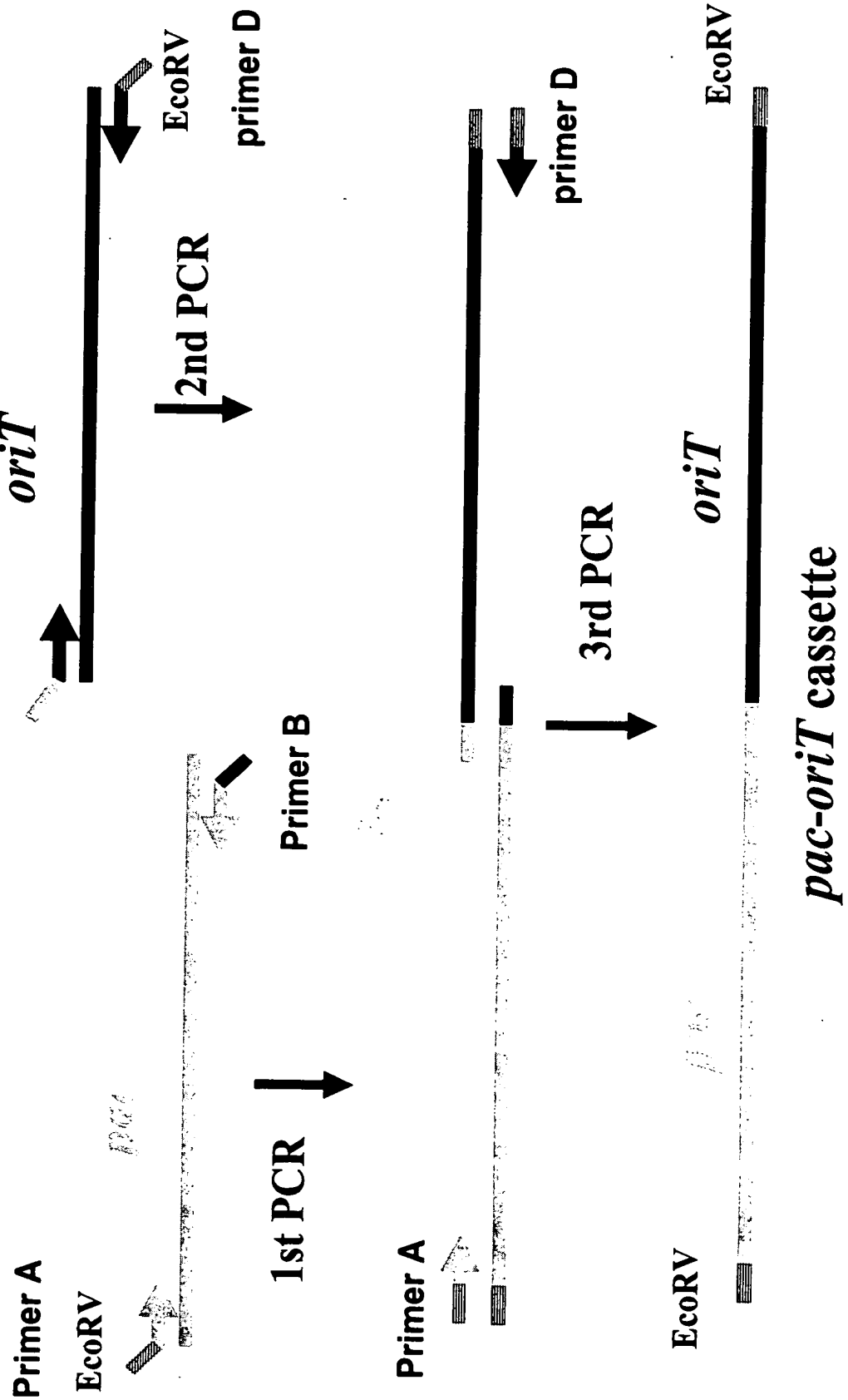

Primer A
EcoRV
1st PCR
Primer B
Primer C
oriT
EcoRV
2nd PCR
primer D
Primer A
3rd PCR
primer D
oriT
EcoRV
EcoRV
pac-oriT cassette

FIG. 29

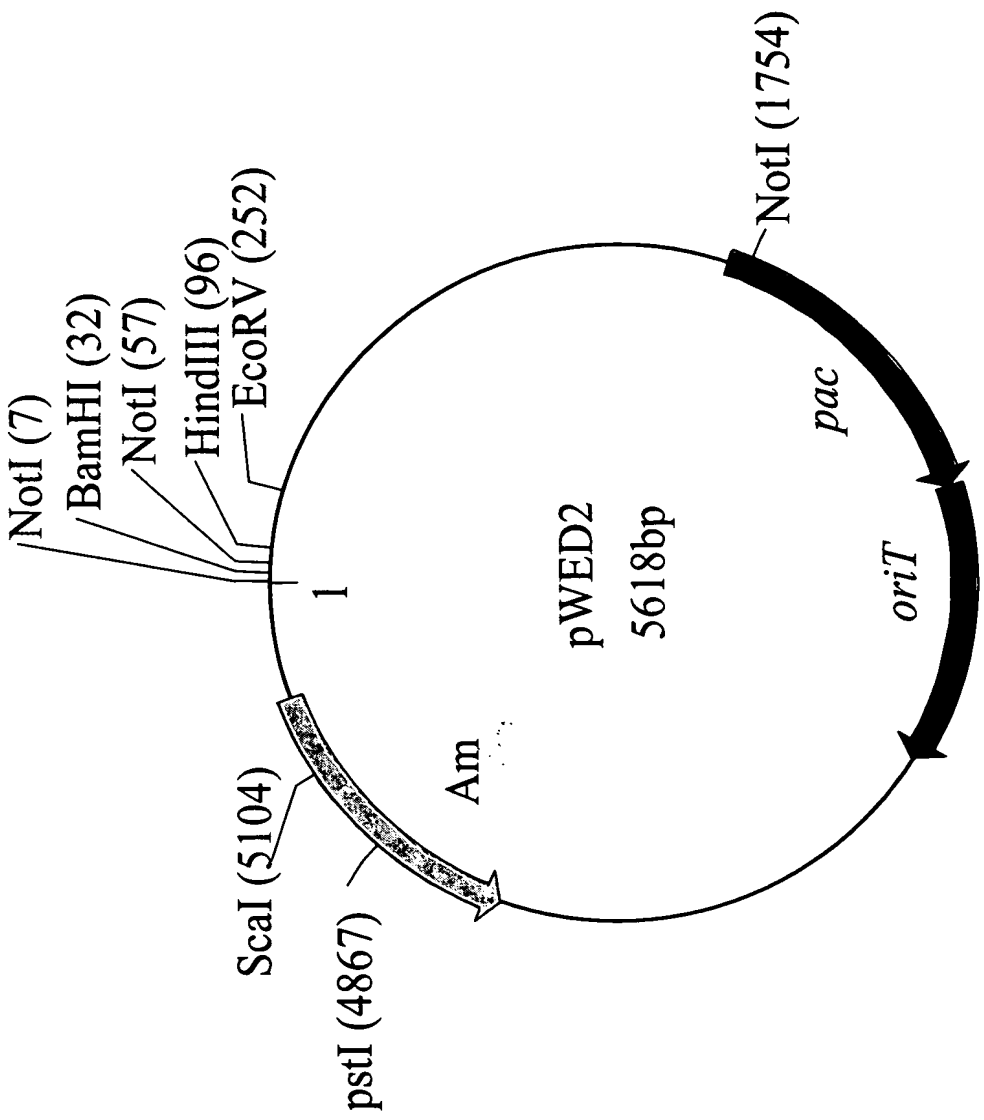
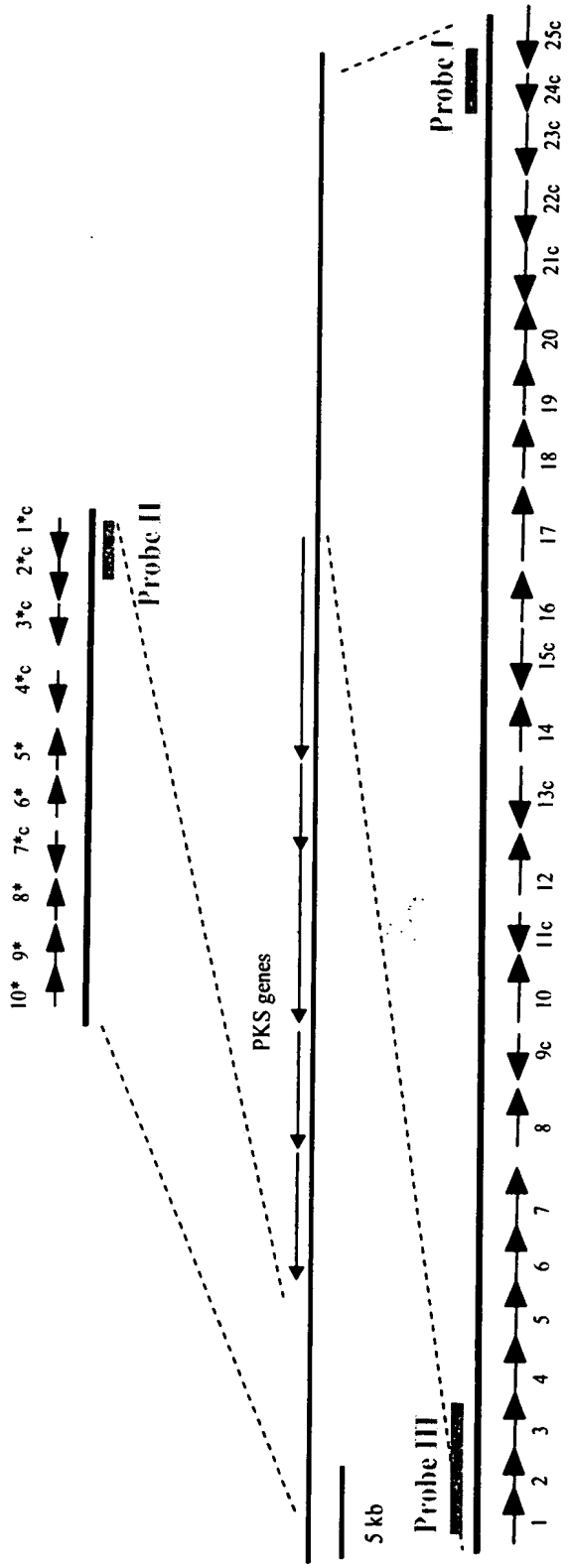

NotI (7)
BamHI (32)
NotI (57)
HindIII (96)
EcoRV (252)
1
NotI (1754)
pac
oriT
pWED2
5618bp
Am
ScaI (5104)
pstI (4867)

FIG. 30



Probe I: PCR product ORF23c-ORF25c

Probe II: PCR product ORF1*c-ORF2*c

Probe III: DNA fragment covering *orf1*, *orf2* and *orf3*

FIG. 31

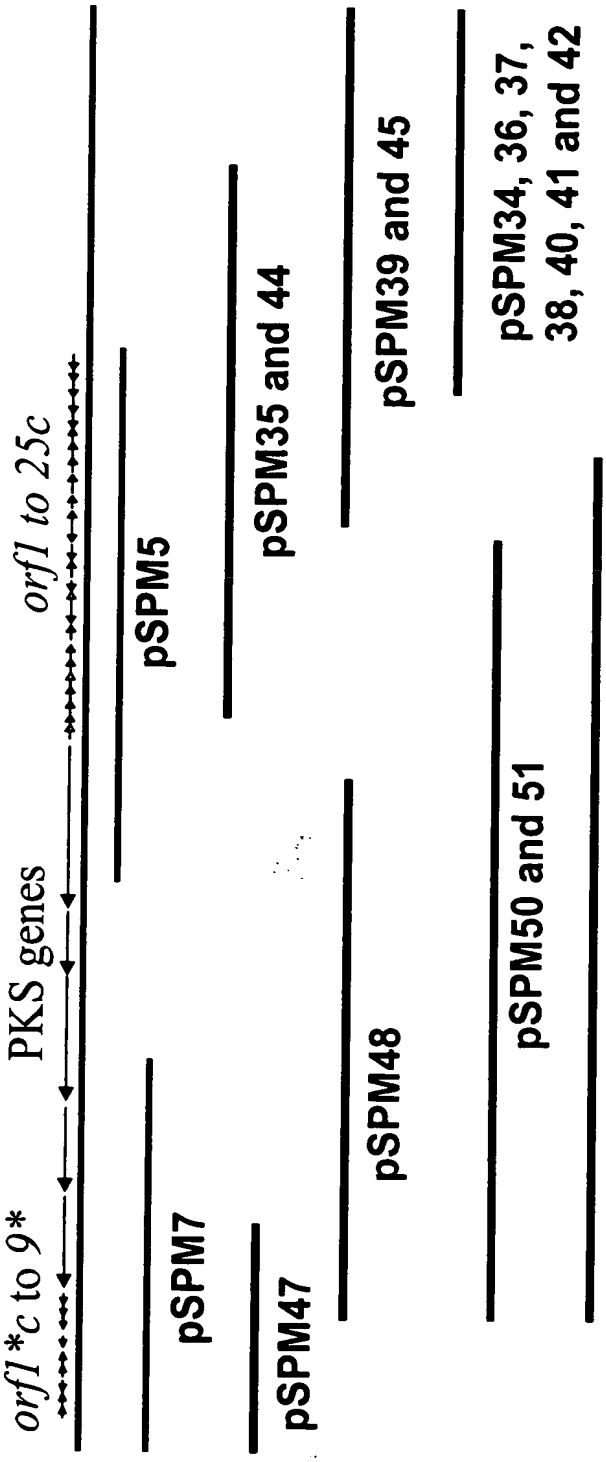

orf1 *c to 9*
PKS genes
orf1 to 25c
pSPM7
pSPM47
pSPM5
pSPM48
pSPM35 and 44
pSPM50 and 51
pSPM39 and 45
pSPM53, 55 and 56
pSPM34, 36, 37, 38, 40, 41 and 42

FIG. 32

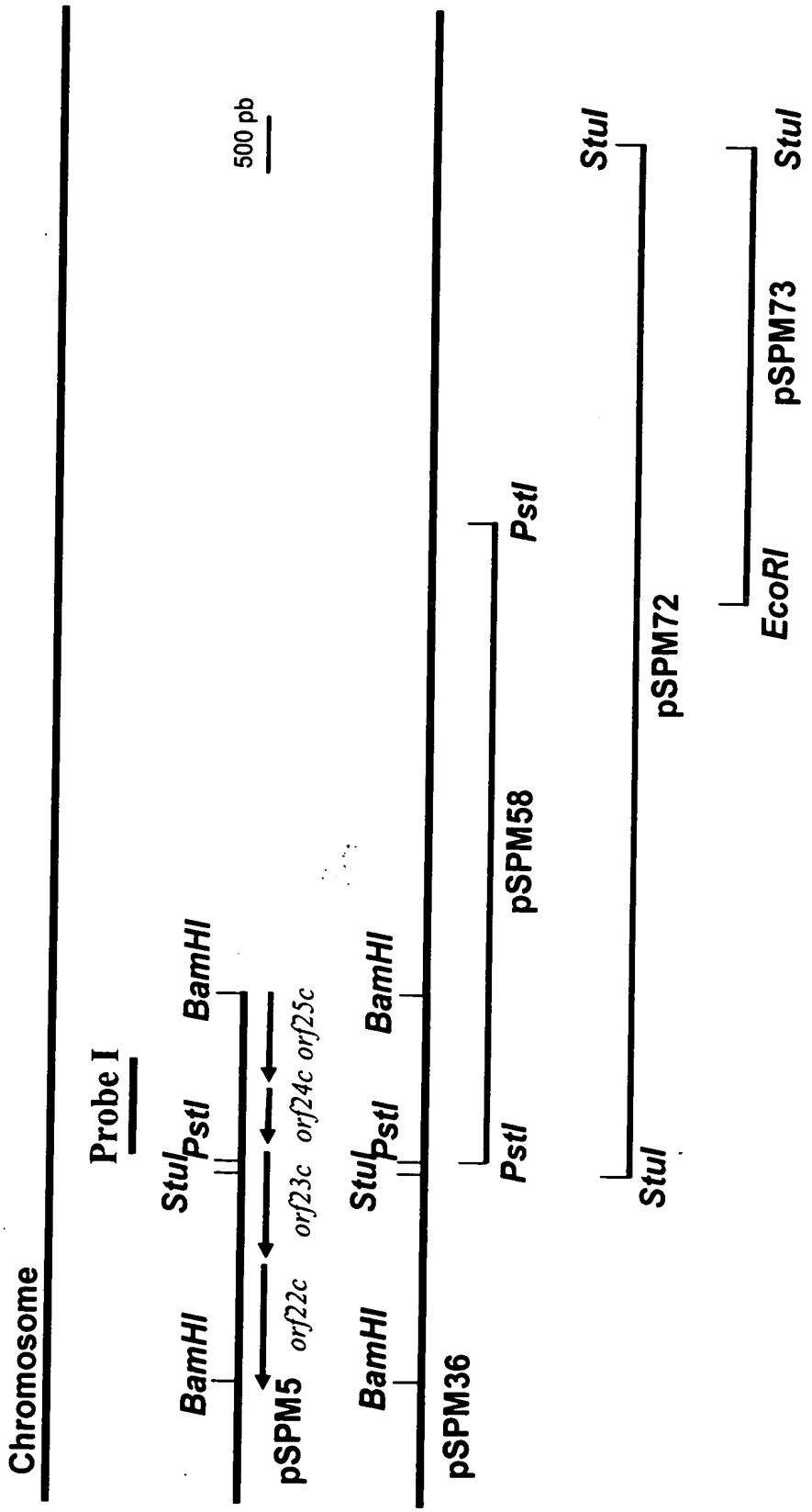

Chromosome
Probe I
500 pb
BamHI
StuI
PstI
BamHI
pSPM5
orf22c
orf23c
orf24c
orf25c
BamHI
StuI
PstI
BamHI
pSPM36
PstI
pSPM58
PstI
StuI
pSPM72
StuI
EcoRI
pSPM73
StuI

FIG. 33

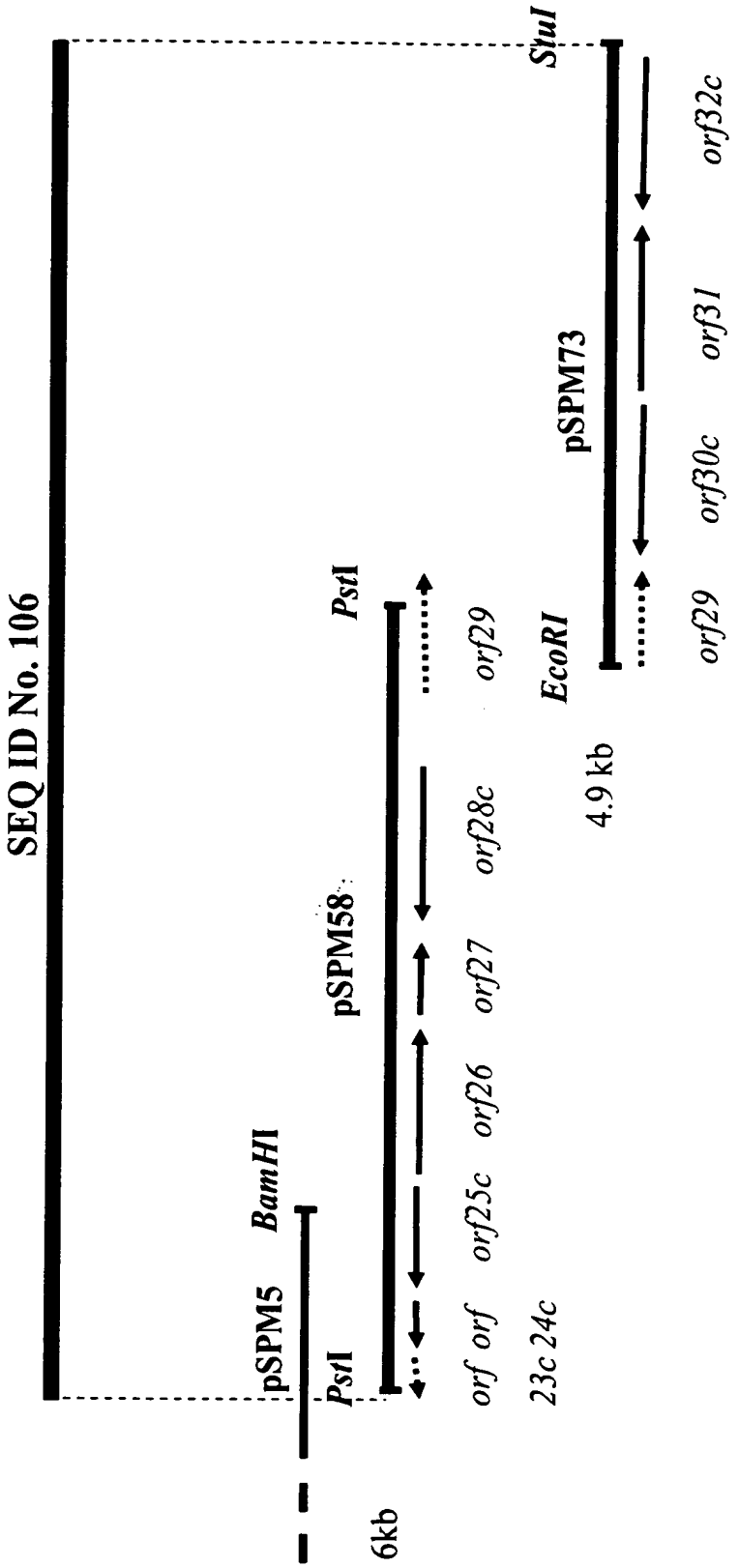

SEQ ID No. 106
pSPM5
BamHI
PstI
6kb
pSPM58
PstI
orf 23c
orf 24c
orf25c
orf26
orf27
orf28c
orf29
EcoRI
pSPM73
StuI
4.9 kb
orf29
orf30c
orf31
orf32c

FIG. 34

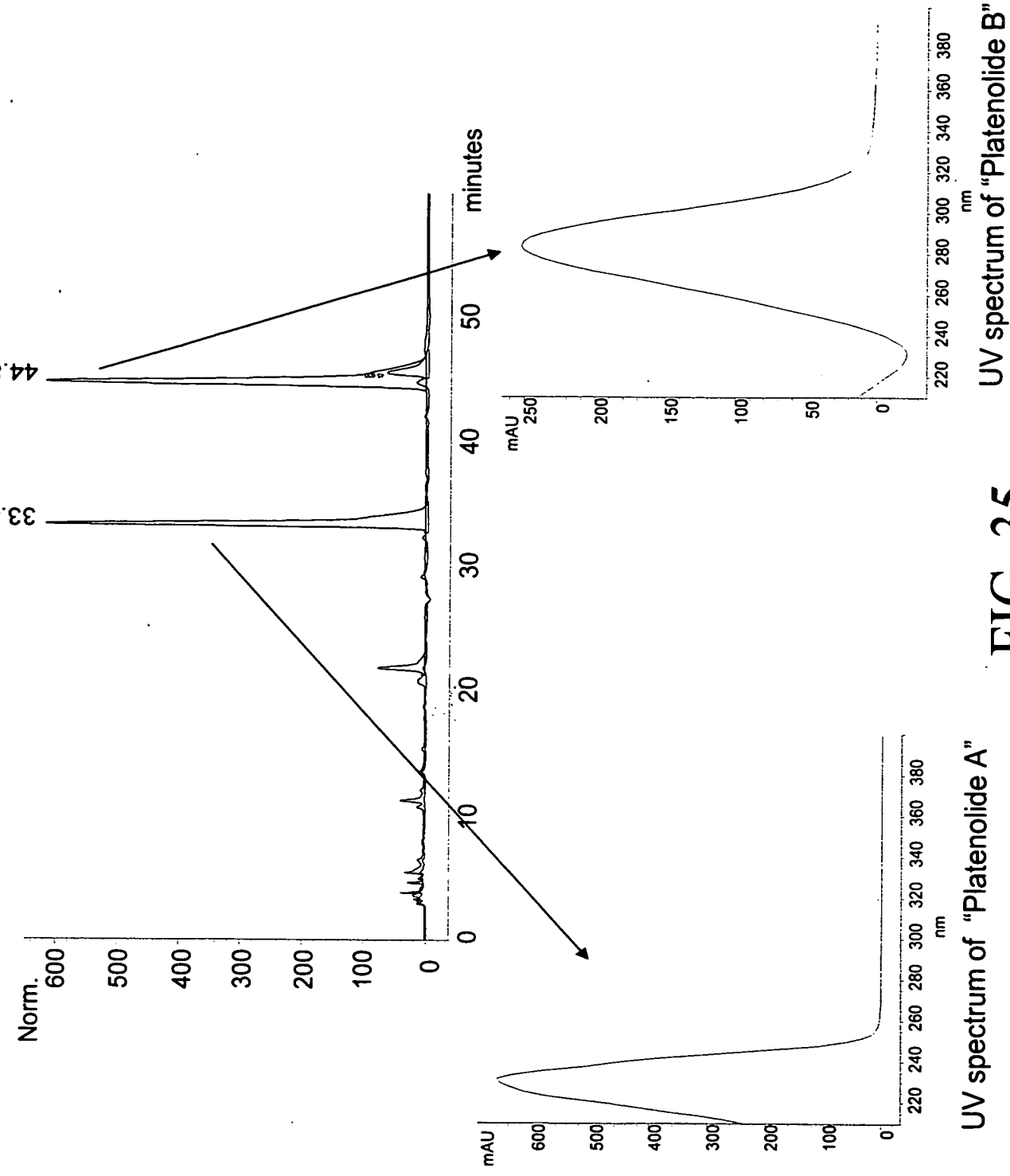
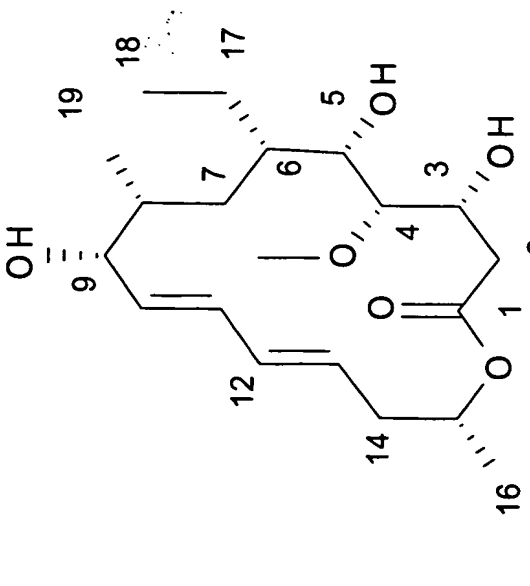

Norm.
600
500
400
300
200
100
0
0
10
20
30
40
50
minutes
33.4
44.8
mAU
250
200
150
100
50
0
220 240 260 280 300 320 340 360 380
nm
UV spectrum of "Platenolide B"
mAU
600
500
400
300
200
100
0
220 240 260 280 300 320 340 360 380
nm
UV spectrum of "Platenolide A"

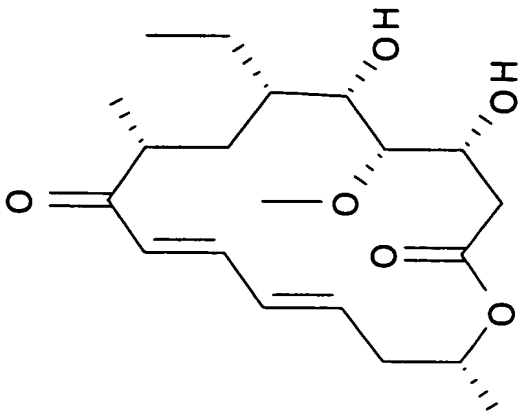
FIG. 35

« Platenolide A »MM =370



Molecular mass =370
Molecular formula=C20H34O6

« Platenolide B » MM=368



Molecular mass =368
Molecular formula =C20H32O6

FIG. 36

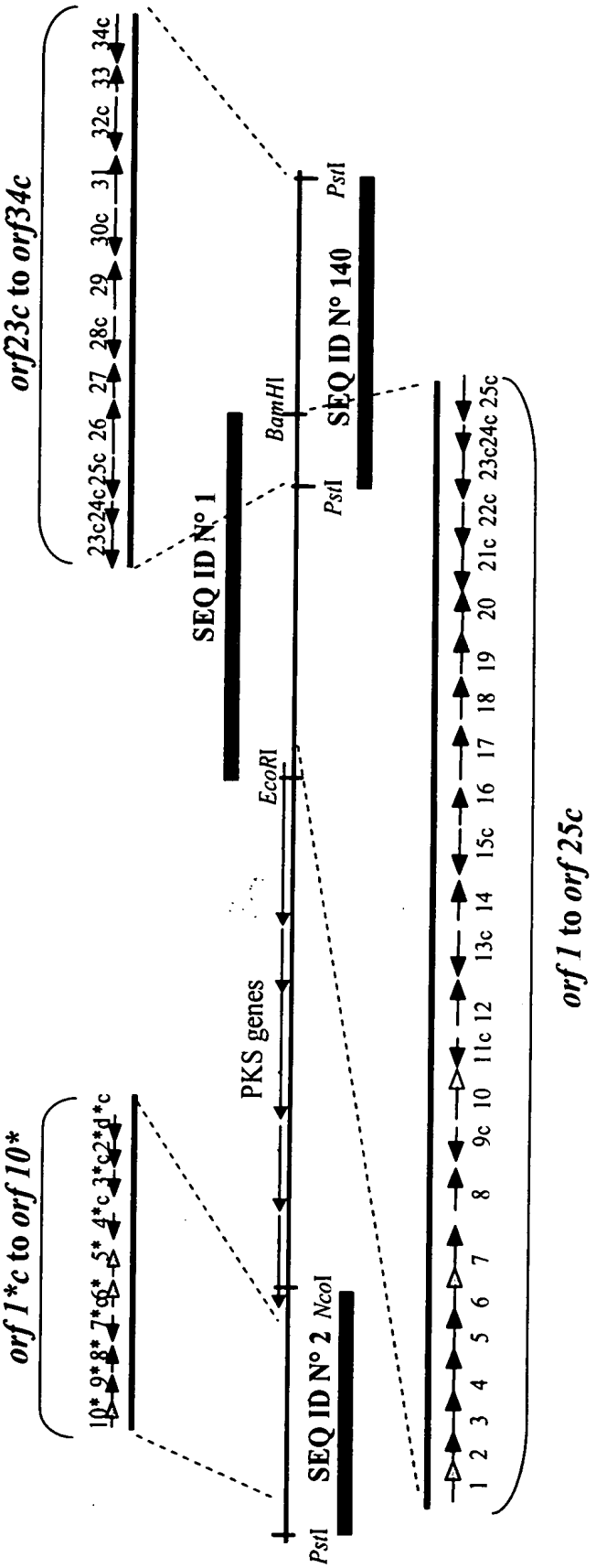

orf23c to orf34c
23c 24c 25c 26 27 28c 29 30c 31 32c 33 34c
SEQ ID N° 1
BamHI
PstI
SEQ ID N° 140
PstI
EcoRI
PKS genes
orf 1*c to orf 10*
10* 9* 8* 7* 6* 5* 4*c 3*c 2*c 1*c
PstI
SEQ ID N° 2
NcoI
1 2 3 4 5 6 7 8 9c 10 11c 12 13c 14 15c 16 17 18 19 20 21c 22c 23c 24c 25c
orf 1 to orf 25c

FIG. 37

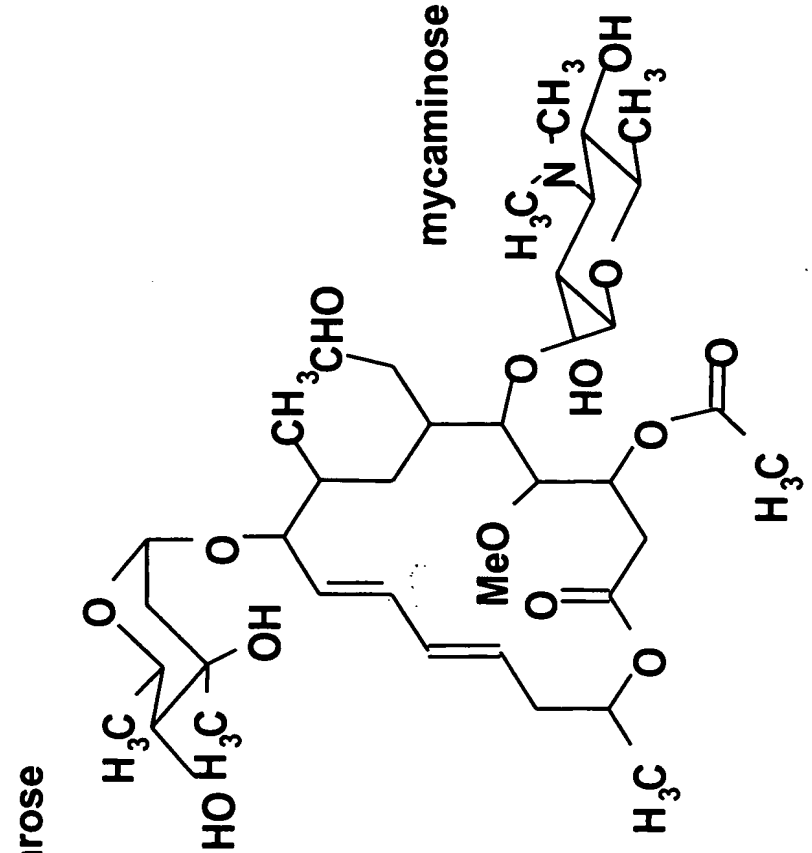


FIG. 38

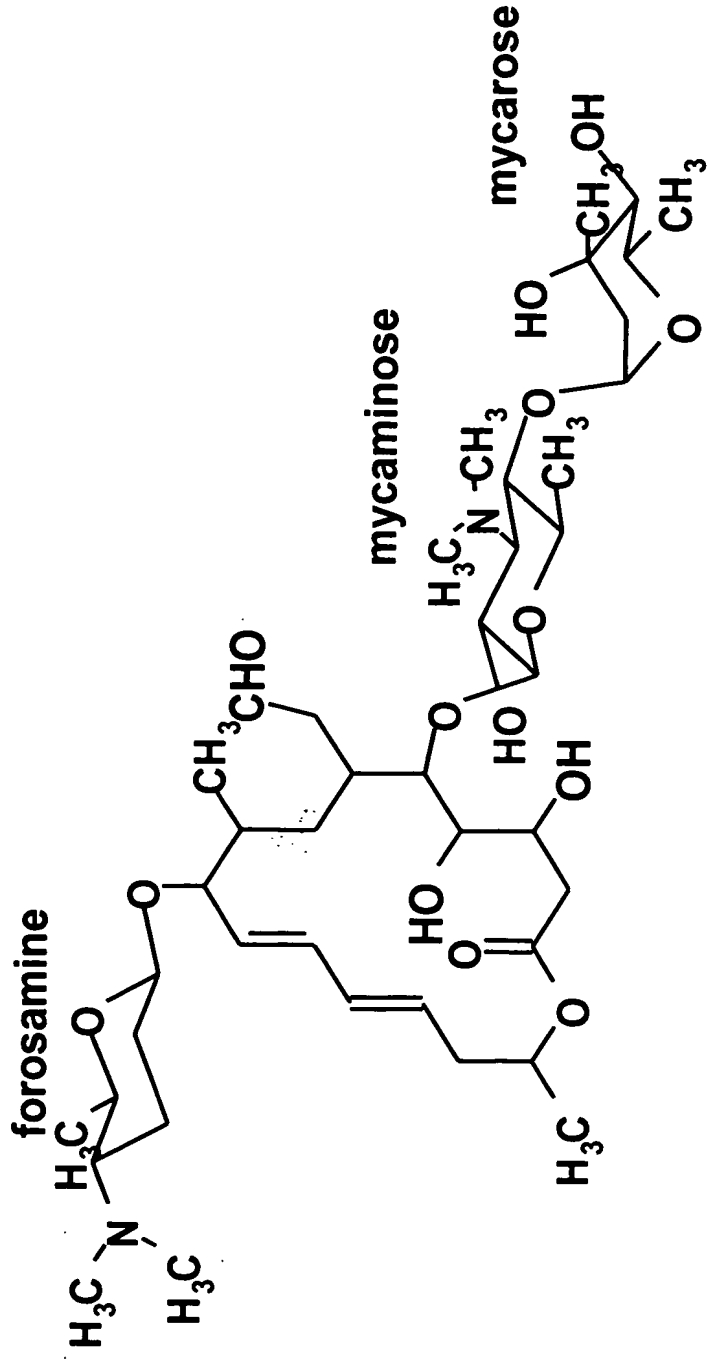
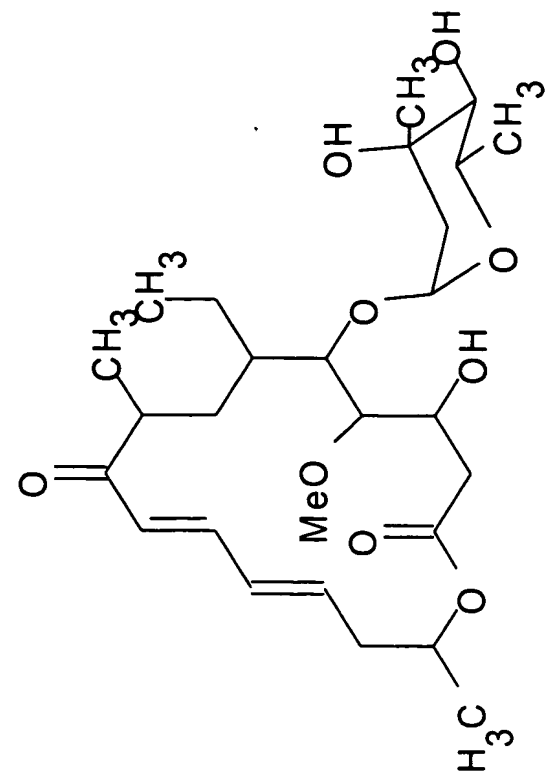
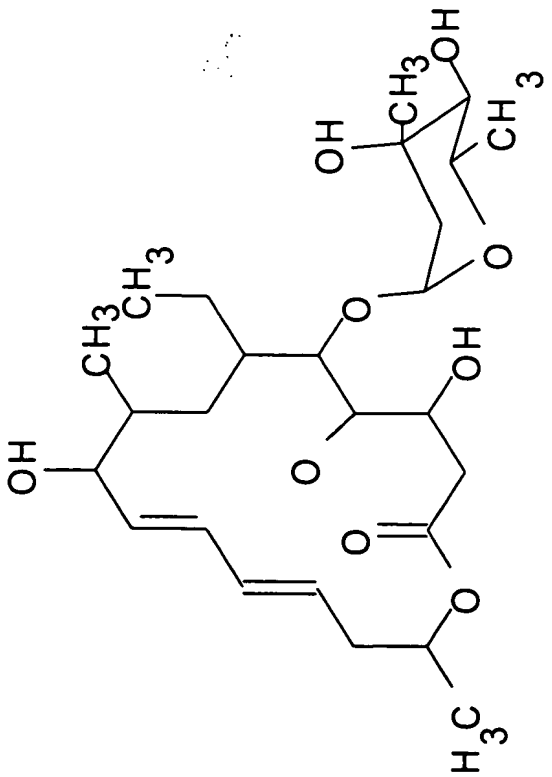

forosamine
mycaminose
mycarose
H3C
N
CH3
CH3CHO
HO
OH
O
CH2OH

FIG. 39



Platenolide B + mycarose



Platenolide A + mycarose

FIG. 40

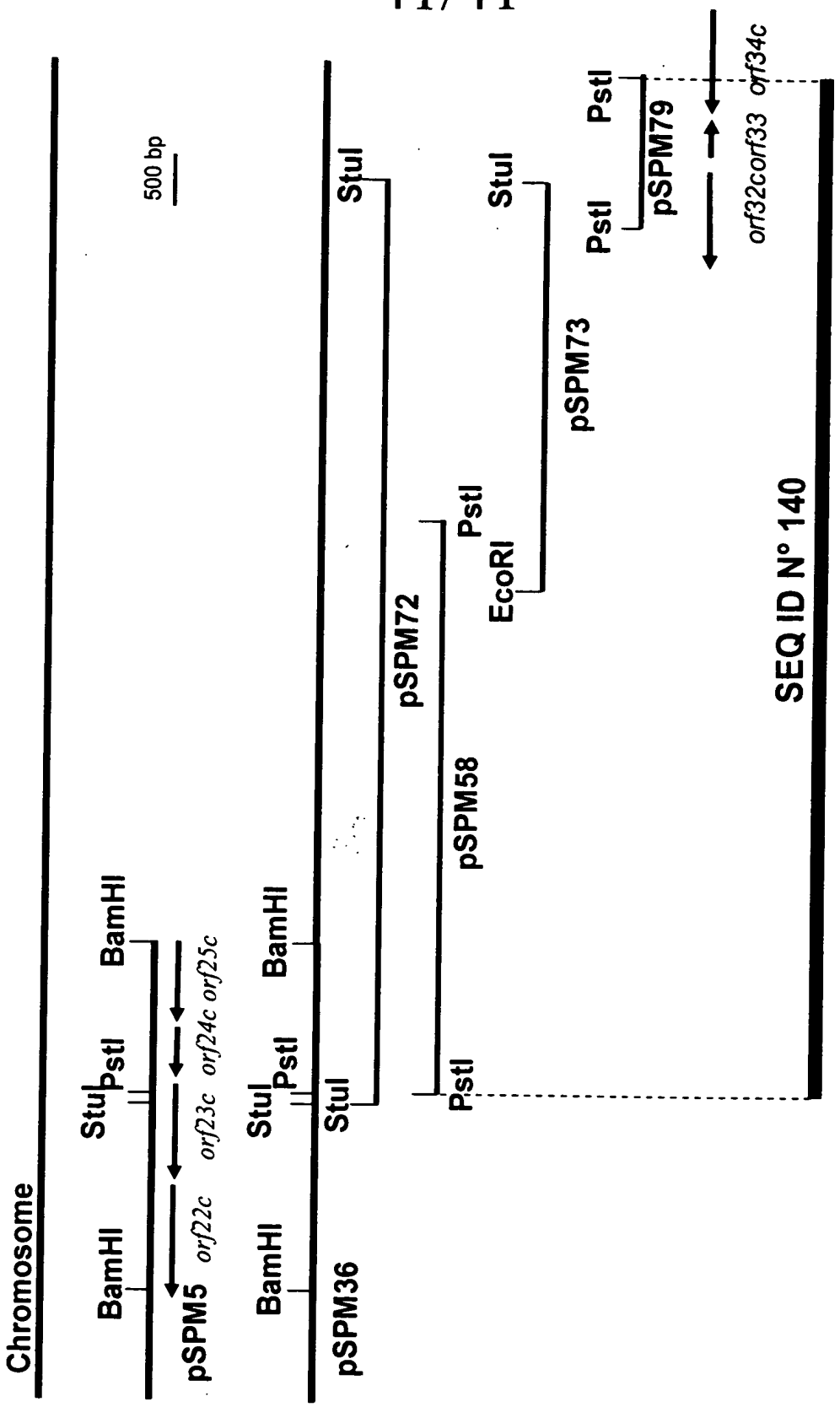

Chromosome
500 bp
BamHI
StuI
PstI
BamHI
pSPM5
orf22c
orf23c
orf24c
orf25c
BamHI
StuI
PstI
StuI
pSPM36
pSPM72
PstI
pSPM58
PstI
EcoRI
StuI
pSPM73
PstI
PstI
pSPM79
orf32corf33
orf34c
SEQ ID N° 140

FIG. 41